TET1-MEDIATED HYDROXYMETHYLATION AND AIRWAY HYPER-RESPONSIVENESS

by
Tyna Dao

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ABSTRACT

Asthma is a complex lung disease characterized by both airway remodeling, which obstructs the flow of air, and chronic airway inflammation. Asthma threatens an individual's ability to breathe through acute airway exacerbations during asthma attacks, which is also known as airway hyper-responsiveness (AHR) to allergic stimuli. Asthma remains poorly understood and difficult to manage, with mostly palliative treatments available, but no effective therapies. Therefore, understanding the mechanisms of asthma pathogenesis is critical to the prevention and treatment of asthma.

Recent studies suggest that epigenetic regulation may, in part, mediate the complex gene-by-environment interactions that can determine the predisposition and severity of asthma. Epigenetic modifications are changes in gene expression that occur in the absence of alterations in DNA sequences, and determine cell, tissue, and organ fates and functions. Aberrant epigenetic mechanisms, such as DNA methylation, are often used as biomarkers in disease states, and are the target of many epigenetic therapies. However, few studies have examined epigenetic changes directly in the lung tissues in the pathogenesis of asthma. Previously, we observed the up-regulation of the ten-eleven translocation methylcytosine dioxygenase 1 (Tet1) protein in mice showing increased house allergen induced AHR. TET proteins are known to convert methylated DNA (5mC) to hydroxymethylated DNA (5hmC); therefore, we hypothesized that the up-regulation of Tet1 contributes to allergen-induced AHR.

This dissertation aimed to investigate the role of Tet1 in AHR, a cardinal feature of asthma, in two distinct models. First, we utilized a mouse model with a heterozygous *Tet1*$^{+/-}$ knockout, and examined their lung and airway smooth muscle cells (ASMCs)

functions after acute house dust mites (HDM) exposure. Second, we translated our findings in the mouse model to human disease by using ASMCs isolated from non-asthmatic and asthmatic lung donors. We aimed to investigate whether TET1 contributes to the aberrant ASMC phenotypes of asthmatics, and why TET1 is dysregulated in asthmatics.

In Chapter 2, we demonstrated that *Tet1* deficiency *in vivo* protects mice from HDM-induced AHR. Our results indicated significant differences in TET activity, global DNA hydroxymethylation, and gene-specific DNA methylation in isolated mouse tracheal ASMCs, suggesting an association between Tet1-mediated hydroxymethylation and the alteration of ASMC phenotypes. Furthermore, we showed that human asthmatic ASMCs exhibited increased TET1 gene expression, TET activity, and global 5hmC content. The knockdown of TET1 by small interfering RNA (*siTET1*) could reverse the aberrant ASMC phenotypes seen in asthmatics. Additionally, in Chapter 3, we demonstrated that TET1 activation and its mediated ASMC phenotypes could be modulated via oxidative stress-dependent pathways.

This dissertation describes the epigenetic regulation in asthma pathogenesis. Since epigenetic changes are potentially modifiable, these studies provide tremendous opportunities for the development of novel interventions for the treatment of asthma.

Advisor:	Winnie Wan-Yee Tang, Ph.D.
Voting Members:	Robert Brown, M.D., M.P.H.
	Wayne Mitzner, Ph.D.
	Anthony Leung, Ph.D.
Alternates:	Alan Scott, Ph.D.
	John Groopman, Ph.D.

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CHAPTER 1

INTRODUCTION

Sections of this chapter have been adapted from a review published in 2014: "Dao, T., Cheng, R.Y.S., Revelo, P., Mitzner. W., and Tang, W.Y. (2014) Hydroxymethylation as a novel environmental biosensor. Curr Environ Health Rep, 2014 Mar 1; 1(1):1-10. PubMed PMID: 24860723."

1.1. Epigenetics is the genome record of exposure

Epigenetic mechanisms are critical responses to the environment, from famine to air pollution, to fine tuning gene expression patterns by modifying the genetic code to effect gene expression regulation, without changing the DNA sequence (1). Therefore, epigenetic mechanisms can interpret environmental exposures and interact with the genome to regulate gene expression and cellular mechanisms, tissue development, and organ physiology. Epigenetic mechanisms are implicated in phenotypic plasticity, or the ability of cells to change their behavior in response to internal and external environmental cues which may disrupt cellular homeostasis and result in the aberrant mechanisms implicated in epigenetic disease (1). Simply stated, epigenetic mechanisms are one answer to how the environment affects diseases, through the alteration of gene expression patterns.

The classic example of a gene-environment interaction mediated by an epigenetic mechanism is from the Dutch Hunger Famine: during World War II, mothers who were starving gave birth to smaller children, which was associated with the hypomethylation of the insulin-like growth factor 2 (*IGF2*) gene (2, 3). The rationale for the modification of the epigenome (the epigenetic genome state) was that a smaller birth weight was advantageous to the newborns, since being smaller required fewer nutrients during

famine conditions. However, complications occur when the *in utero* environment does not match the environment at birth; thus, epigenetic reprogramming occurs based on environmental cues, which results in alterations in gene expression patterns and an increased risk of epigenetic disease. After World War II, Dutch Hunger Famine infants had excess nutrition, and epidemiological evidence associated the cohort with increased schizophrenia (4) and cardiovascular disease (5) compared to the control populations.

There is also evidence that prenatal exposure to cigarette smoke (6, 7), polycyclic aromatic hydrocarbons in air pollution (8), arsenic (9), and endocrine disrupting chemicals (9, 10) may have effects on the epigenetic outcomes and disease susceptibility in offspring. Another classic study of a gene-environment interaction comes from monozygotic twin studies, in which the genetic determinants were identical but the environments varied (11, 12). Different lifestyle choices, or environments, made each twin more or less susceptible to chronic disease states, suggesting that certain environmental exposures were interacting with genetic determinants to shape their disease susceptibility. Therefore, epigenetic reprogramming can trigger either somatic or germ line changes in the epigenome, which can affect disease susceptibility during development, and may be transmitted through multiple generations (13).

There is increasing epidemiological evidence that environmental exposures can alter the epigenetic mechanisms in somatic cells during development that are associated with altered risks of disease. By understanding the unique "signatures", of environmental exposure upon the epigenome, we can gain insight into the biological mechanisms that are aberrant in disease development, and the opportunities to therapeutically intervene. In order to define the epigenetic "signatures," we must identify the exposure, which is

extremely difficult in cross-sectional epidemiological studies. Nevertheless, epidemiological studies provide valuable insight into the aberrant epigenetic mechanisms associated with disease risk, although these mechanisms are often considered to be causal associations, and the exact mechanisms have not yet been established (14). There has long been an association between the regional composition of ambient air pollutants and the risk of asthma, including ambient nitric oxide (15), ozone (16), suspended particulate matter (14), diesel exhaust phthalates (15, 16), and polycyclic aromatic hydrocarbons (PAHs) (8). In addition to air pollution, epidemiological studies have discovered an association between arsenic and the exposure to heavy metals and cardiovascular health risks (17, 18). These epidemiological studies demonstrate the role of epigenetic mechanisms associated with the gene-environment interactions implicated in disease development. Therefore, studies into the role of environmental exposures upon epigenetic mechanisms provide novel insights into disease pathogenesis, therapeutic strategies, and public health interventions.

1.1.1. Mechanisms of epigenetic regulation

Epigenetic mechanisms alter gene expression without changing DNA sequences, which means that epigenetic mechanisms act “above” or “on top” of genetics to alter gene transcription. This ability to modulate transcriptional programs determines the cell identity, regulates molecular mechanisms, and modifies physiological responses. Therefore, aberrant epigenetic mechanisms lead to disruptions in gene expression patterns, which can contribute to altered phenotypes and disease pathogenesis.

There are three main epigenetic mechanisms: DNA methylation, histone modifications, and microRNAs. The epigenome is composed of combinatorial events from all three epigenetic mechanisms, and as such, the study of epigenetic mechanisms with regard to transcriptional regulation is complex.

DNA is compactly organized into nucleosomes, and in order for the transcriptional machinery to translate DNA, the chromatin structure must be accessible. Therefore, modifications (acetylation, phosphorylation, and methylation) of the histone tails can recruit transcription factors and enzymes to open or close the chromatin structure to regulate gene transcription. Once the DNA sequence is accessible, DNA methylation can further regulate the gene expression patterns. DNA methylation occurs in CG dinucleotides, usually in CpG islands, to convert the 5' of cytosine to 5-methylcytosine (5mC). DNA methyltransferases (DNMTs) are enzymes that are essential to introducing a methyl group, from an **S-adenosylmethionine** substrate, onto a DNA strand to produce 5mC. DNMT3a and DNMT3b are for *de novo* 5mC synthesis, while DNMT1 is required to maintain and propagate the modification of DNA methylation during DNA replication (19). DNA methylation or 5mC regulates gene transcription through three known mechanisms: 1) 5mC is recognized by methyl-CpG-binding domain (MBD) proteins, which further recruit histone modifiers to form complexes to silence gene expression; 2) 5mC can also inhibit transcription factor binding in the promoter region, and inhibit gene transcription; and 3) 5mC can block the transcriptional machinery (RNA polymerase) to inhibit gene transcription (20). Although DNA methylation is generally considered to be repressive, there are exceptions. Certain CpG dinucleotides are more essential to gene transcription then others, such as those critical

for transcription factor binding or transcriptional machinery. Therefore, DNA methylation is generally considered to be repressive, and the absence or loss of methylation would permit active gene transcription.

In summary, both histone modifications and DNA methylation regulate the direct transcription of genes from the DNA sequence, by modifying the histone structure and transcription factor binding. However, once the genes are transcribed into mRNA sequences, microRNAs are able to suppress or silence gene expression, targeting specific mRNA sequences. Epigenetic mechanisms act upon the DNA by altering the histone structure to allow or prevent access to the DNA sequence, modifying the DNA sequence through DNA methylation to adjust transcriptional regulation and targeting mature mRNA sequences through microRNAs. Some combination of these epigenetic mechanisms is essential to regulate gene expression patterns for developmental biology, since all cells are derived from the same DNA sequence, yet have vastly different functions. Consequently, once the cell fate is determined, epigenetic mechanisms also occur in response to environmental cues, and these perturbations in the epigenome can disrupt the gene expression patterns implicated in disease mechanisms. Epigenetic mechanisms are modifiable, or reversible, so understanding these mechanisms in disease pathogenesis provides novel therapeutic opportunities.

1.1.2. Therapeutic potential of epigenetic therapy

Disease susceptibility depends on the dynamic interaction between an individual's inherent genetic predisposition and their acquired epigenetic transcript. Overall, epigenetic drift suggests that as mammalian cells age there is a gradual global decrease in

DNA methylation patterns, resulting in aberrant gene expression and instability, which leads to increased disease susceptibility (13). For example, since the pathogenesis of cancer occurs over a lifetime, and usually manifests in older individuals, there have been extensive investigations into the epigenetic mechanisms of cancer regulation. Therefore, the use of epigenetic drugs or "epidrugs" has been attempted in cancer research to target aberrant epigenetic mechanisms. The United States Food and Drug Administration (FDA) has approved two DNMT inhibitors, 5-azacytidine (Vidaza) and 5-aza-2-deoxycytidine (Decitabine), to treat myelodysplastic syndromes and three HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) (Vorinostat) and depsipeptide (Romidepsin), to treat cutaneous T-cell lymphomas, and the antiepileptic drug valproic acid for the treatment of leukemia, breast, and ovarian cancer (21). More epidrugs are currently in Phase I/II clinical trials for cancer and other disorders (13, 21). Epigenetic mechanisms are also being investigated in cancer therapy to predict drug absorption, distribution, metabolism, and excretion characteristics, as well as predict acquired drug resistance in chemotherapy (21). Therefore, epigenetic differences can elucidate inter-individual variations in drug response and therapy, and also provide unique opportunities for personalized medicine tailored towards epigenetic abnormalities.

Small molecule epidrugs have been effective in reversing aberrant epigenetic mechanisms implicated in disease pathogenesis, and in improving disease outcomes. This success has prompted further research into the epigenetic mechanisms underlying other disease mechanisms, and the opportunity for epigenetic intervention to alter the outcomes of disease.

1.2. Asthma and epigenetics: Public health relevance

Asthma is a chronic inflammatory disorder of the airways, coupled with airway remodeling, which restricts the airflow and increases hyper-reactivity to allergens, resulting in increased difficulty in breathing. Asthma has become increasingly common and a growing proportion of severely asthmatic patients have not been responding to the current therapeutic options. Thus, the investigation of the epigenetic mechanisms implicated in asthmatic disease provides unique opportunities for therapeutic intervention.

1.2.1. Asthmatic disease and treatment review

Asthma is public health concern, affecting approximately 25 million people in the United States (235-300 million people worldwide), accounting for an estimated $18 billion in the US for both direct and indirect costs (22, 23). The current understandings of asthmatic disease are that it is a chronic respiratory disorder with increased airway hyper-responsiveness (AHR) causing airway narrowing, resulting in obstructed airways and the clinical manifestations of wheezing and the shortness of breath (25). Pathologically, both chronic inflammation and airway remodeling define asthma. The inflammatory immune response plays critical and divergent roles that affect many cell phenotypes, resulting in the hallmark increased eosinophilic inflammation of asthma. Although there is increasing evidence for the role of neutrophilic driven inflammation (23), we will focus mainly on the immune mechanism of eosinophilic inflammation.

Asthma is also attributed to structural changes, or airway remodeling, that interfere with airflow. The airway remodeling consists of airway walls that are thickened through increased extracellular matrix protein deposition (such as collagen and elastin), increased angiogenesis, and increased airway smooth muscle (ASM) mass (26). There is constant debate whether airway inflammation or airway remodeling occurs earlier, and which mechanisms are fundamental to asthma disease pathogenesis (26, 27).

In one study of patients with asthma exposed to house dust mite allergen or methacholine (MCh), a bronchoconstriction agent, found that in the absence of allergen induced eosinophilic infiltration, both the allergen and MCh challenged lung biopsies of the patients exhibited increased collagen deposition and epithelial goblet cell formation (25). This is one of many studies highlighting the important role of bronchoconstriction on the structural airway changes seen in airway remodeling, rather than the inflammatory immune response upon the asthma pathogenesis in patients. Decades of research and therapeutic strategies have provided only palliative treatments, but no cure for asthma, suggesting that both airway inflammation and airway remodeling are interdependent processes underlying the clinical features of asthma (26). Therefore, a prevalent mechanism for asthma involves a persistent pro-inflammatory immune response in the large airways, which recruits and activates inflammatory cells, cytokines, and mediators that target structural changes in the epithelial cells and airway smooth muscle (28, 29). The myriad of immunological and structural changes encompassing asthma pathogenesis complicates the success of therapeutic intervention strategies in asthma treatment. Therefore, clinicians in the field have begun to characterize the asthma endotypes or

different asthma phenotypes attributable to specific pathophysiological mechanisms, in order to personalize therapeutic interventions (23).

The endotypes of asthma depend on the trends regarding the key Th2 cytokines (IL4, IL5, and IL13) and eosinophils in the blood and tissues (23). The most widely accepted paradigm for the increased inflammation in asthma involves a skewed pro-inflammatory Th2 phenotype and the diminution of the anti-inflammatory Th1 phenotype. The Th2 phenotype presents itself histologically as increased eosinophils, mucosal mast cells, and activated T cells in the asthmatic airways (29). The inhalation of antigens into the airways interacts directly with the airway epithelium, the first barrier of defense. Under normal conditions, the cells in the epithelium provide protection by inhibiting the penetration of the antigen, while also clearing the antigen through mucus secretion and mucociliary clearance. However, under increased allergen exposure, the epithelial barrier can be damaged through direct (protease activity of allergen) and indirect mechanisms, upon which the airway epithelial cells produce distress cytokines or "alarmins," such as interleukin 33 (IL33), thymic stromal lymphopoietin (TSLP), and interleukin 25 (IL25), to recruit and activate dendritic cells, innate lymphoid cells, and basophils (23). These signals and antigens are recognized and captured by antigen-presenting dendritic cells and presented to naïve $CD4^+$ T cells, which then differentiate and proliferate into specific lineages or subsets of the $CD4^+$ T helper cells (Th1, Th2, Th17, or T regulatory cells) that synthesize and secrete specific cytokines to signal a specialized immune response. Therefore, the interaction between the epithelial cells and dendritic cells is crucial for initiating the Th2 immune response (23).

The skewed differentiation and activation of the Th2 lineage cells results in the release of the pro-inflammatory cytokines implicated in the pathogenesis of asthma: IL5, IL13, and IL4 (28). The IL5 cytokines released from the Th2 cells primarily recruit eosinophils into the airways, and interact with the IL5 receptors on the cell surface of eosinophils to increase eosinophil activation, survival, and proliferation, which increases the eosinophilic expression of transforming growth factor beta (TGFβ) (23, 30, 31). TGFβ acts upon the myofibroblasts to increase collagen deposition, and smooth muscle to increase proliferation (30).

Another pleiotropic cytokine, IL13, is released from the Th2 cells, airway smooth muscle, and airway epithelium, and up-regulated in asthmatic patients (32). IL13 has been found to act upon the airway epithelium to induce mucus hyperplasia and subepithelial fibrosis (23, 27, 32). IL13 and IL4 also promote IgE isotype switching in B cells (23, 28, 29). The plasma B cells then synthesize and release antigen specific IgE that binds to the mast cells and activates the release of histamine and other bronchoconstrictors, which recruit eosinophils into the airways (28, 29, 32). The mast cells also release IL4, IL5, and IL13 cytokines to amplify the proinflammatory immune response (29). IL4 further amplifies the immune response, since it is required to prime and mature the Th2 cells for differentiation and cytokine production (23, 28). In summary, the mediators and cytokines from the inflammatory immune response act upon epithelial cells, myofibroblasts, and airway smooth muscle to contribute to the structural changes involved in airway remodeling. Consequently, the Th2 inflammatory immune response is a common and dynamic phenotype of asthma patients, with a wide range of mediators, effectors, and targets that complicate asthma treatment.

The treatment of asthma has remained largely unchanged for decades. There are two predominant treatments: 1) inhaled bronchodilators (short or long acting beta2-agonists) that relax smooth muscle and 2) inhaled corticosteroids, which reduce inflammation (22, 23, 28). The routine adherence to these treatments is effective in 90-95% of the population; but for severe asthmatics, these treatments even at maximum doses are not effective (22, 23, 29). Although beta2-agonist therapy is routinely used, desensitization can occur over time, due to the loss of beta adrenergic receptors on the cell surface, and result in severe or fatal asthmatic attacks (32). Also, there is conflicting evidence for the clinical effectiveness of corticosteroids to reduce airway remodeling (30). Interestingly, the combined use of corticosteroids and beta agonists is often prescribed, since corticosteroids have been shown to increase beta adrenergic receptor expression, and therefore, increase the effectiveness of beta2-agonists in relaxing smooth muscle (32).

For severe asthmatics, these two drugs are frequently insufficient; therefore, endotype specific therapies have been introduced to curtail the inflammatory immune response in more personalized asthma therapy and management (22). For example, Omalizumab (Genentech/Novartis), an anti-immunoglobulin E (anti-IgE) monoclonal antibody, has been used in patients in clinical trials, but the results vary (22). In addition, Lebrikizumab (Genentech), an anti-IL13 antibody, has been shown to increase FEV_1 in asthmatic patients, indicating improved asthma control (22, 23). Dupilumab (Regeneron) (an anti-IL4 receptor alpha subunit antibody), Mepolizumab (GlaxoSmithKline) (anti-IL5 antibody), and Benralizumab (AstraZeneca/MedImmune) (anti-IL5 receptor antibody) have been shown in ongoing clinical trials to improve the asthma symptoms of a specific

subset of severe asthma patients (22, 23, 31). The anti-IL5 antibody administered to asthmatic patients resulted in decreased airway eosinophils, and decreased TGFβ expression associated with decreased extracellular matrix remodeling proteins (30). However, anti-IL5 administration decreased the eosinophilic infiltration by only 40-50% in the airways of asthmatic humans, resulting in mixed therapeutic benefits, indicating that there are alternative pathways to recruit, activate, and maintain eosinophils in the airways (30, 31). The role of innate lymphoid cells, upon activation by allergen exposure at the airway epithelium have been found to proliferate and release IL5 and IL13 and are being recognized as driving the eosinophilic asthma phenotype, independent of the Th2 response (23). These inflammatory immune response therapies have been shown to be moderately successful in specific endotypes of allergic asthma patients only, due to the complex heterogeneity that complicates the clinical classification of asthma phenotypes (22, 23, 28, 31). With regard to airway remodeling treatments, bronchial thermoplasty to ablate ASM mas, has been identified in experimental models to alleviate the clinical symptoms of asthma in dogs (22). In clinical trials, human airways treated at 65°C showed a 50% reduction in the airway smooth muscle mass, but no improvement in the pulmonary function, as measured by FEV_1 (22).

There have been many therapeutic approaches to asthma control, especially regarding immunomodulation, but they have had limited success in the treatment of asthma (29). Therefore, by further understanding the molecular mechanisms of asthma pathogenesis, coupled with the improved characterization of the clinical phenotypes of asthma, there are many opportunities to improve therapeutic intervention.

1.2.2. Epigenetic regulation in asthma pathogenesis

To further complicate asthma disease mechanisms, the etiology of asthma involves the interactions of both genetic and environmental components. Predominantly, the epigenetic mechanisms involved in asthma pathogenesis have been investigated in experimental mouse models of allergic asthma. Ovalbumin, derived from chicken eggs, has been ubiquitously used as an experimental allergen to induce asthma in many laboratory animal models (mouse, rat, and guinea pig) (33), because an ovalbumin challenge invokes the increased eosinophilic inflammation, increased bronchoconstriction, and airway remodeling fundamental in the clinical features of asthma (33). Despite these experimental strengths, ovalbumin has no human relevance since environmental ovalbumin exposure is practically nonexistent, and therefore, not implicated in asthmatic disease.

Environmentally relevant allergens, such as house dust mites (HDMs), are becoming more common in inducing mouse models of asthma. HDMs are considered to be the most prevalent allergen (34), and are pervasive in household carpets and mattresses with the two most common house dust mite species being *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farina* (35). Clinically, increased HDM-specific IgE in the serum of asthmatic patients (36) supports the role of HDM induced allergies. Cohort studies in Taiwan reported that more than 80% of asthmatic patients were found to be allergic to the Dp species of HDM (measured by HDM-specific IgE) (35). Moreover, HDM extract in mouse models of asthma has revealed increased eosinophilic driven lung inflammation and AHR (35, 37-39), similar to the ovalbumin mouse models that portray the clinical features of asthma. The commercially available

HDM extracts commonly used in mouse models contain a whole mite crushed mixture and fecal matter comprised of over 20 proteins or allergic components (35, 39), including endotoxins, proteases, and chitinases. These invade through the epithelial barrier to promote allergic sensitization, either directly through protease activity or indirectly by activating epithelial cell mediators that prime the immune response described above. The commercially available HDM extract does not precisely characterize the allergic composition of inhaled house dust mites in human exposure, since the HDM composition varies widely depending on the source and time of exposure (39). However, the HDM extract exposure does provide an environmentally relevant allergen, compared to ovalbumin, to examine the possible epigenetic mechanisms implicated in the pathogenesis of asthma.

There have been many investigations into the epigenetic mechanisms underlying asthma pathogenesis (40, 41), most often focused on the modulation of the inflammatory immune response. The roles of epigenetic mechanisms, such as DNA methylation, have been implicated in the differentiation and regulation of T cells and B cells (42). In epidemiological studies, differential DNA methylation profiles are commonly investigated epigenetic mechanisms with regard to the risk of asthma. For example, increased urinary levels of phthalates (a commonly used plasticizer) were associated with an increased asthma risk in children, and correlated with the decreased DNA methylation of TNF-alpha and increased TNF-alpha protein (43).

The exposure to polycyclic aromatic hydrocarbons (PAHs), formed from incomplete combustion and inhaled from polluted ambient air, was associated with

FOXP2 hypermethylation and decreased regulatory T cell (Treg) function associated with asthmatic children (8). Another study of African American inner-city children found that the peripheral blood mononuclear cells (PBMCs) in the circulating blood revealed many differentially methylated regions (DMRs), in which 80% of the DMRs were hypomethylated, including the crucial Th2 related genes IL4 and IL13; these hypomethylated DNA regions were associated with increased gene expression (24). However, clinical evidence cannot prove whether the DNA methylation, histone modifications, or miRNA differences observed between normal and diseased patients are prerequisites or manifestations of the pathogenesis of the disease (42, 44). Therefore, animal models are used to further investigate the epigenetic mechanisms and interventions to improve asthma therapy.

Allergic animal models have shown that allergen exposure can modify the epigenetic mechanisms linked to disease phenotypes. Chronic ovalbumin exposure in C57B6 mice induced the expected asthma phenotypes, including increased AHR, airway inflammation, and airway remodeling, but the combined treatment of ovalbumin with valproic acid, an FDA approved HDAC inhibitor, resulted in decreased ovalbumin induced AHR, as well as decreased epithelial thickness and Tgfβ1 expression in mouse lungs (20). These results indicate that HDAC inhibition can reverse the epigenetic modifications from ovalbumin exposure, and provide protection from AHR, possibly by dampening the effects of Tgfβ1 on epithelial cell growth. In addition, our lab has shown that both acute (38) and chronic (45) HDM exposures in C57B6 mice have been shown to induce AHR and the associated phenotypes, as well as many differentially methylated genes and the aberrant gene expression related to AHR phenotypes. These are some

examples of the current use of animal models to elucidate epigenetic mechanisms.

Corticosteroid treatments, routinely used to suppress inflammation in asthma control, work by recruiting histone deacetylase 2 (HDAC2) to multiple activated inflammatory genes, in which HDAC2 removes the acetylation chromatin modifications to abrogate the expression of the inflammatory genes (29). Therefore, asthma patients with corticosteroid resistance have impaired HDAC2 activity (29). *In vitro*, treatment with the drug theophylline has been shown to increase HDAC2 activity, and possibly increase the responsiveness to corticosteroid treatment (29). Therefore, with increased knowledge of the aberrant epigenetic mechanisms among patients, there is an increasing shift towards using epidrugs in personalized medicine, based on an individual's epigenome (46).

There is preliminary data from animal models that the inhibition of DNA methylation with 5-azacytidine, non-selective HDAC inhibition with trichostatin A, and knockout of mir-155 in ovalbumin sensitized mice have all demonstrated reduced asthma phenotypes, indicating the potential for epigenetic modulation in novel asthmatic therapy (44). There is overwhelming laboratory research and epidemiological evidence that suggests the diverse epigenetic roles implicated in asthma pathogenesis. However, since there is still no successful therapy for severe asthmatics, there is a lot of promise for epigenetic therapies for asthma; but first, we must thoroughly investigate the mechanisms responsible for the disease pathogenesis.

1.3. A novel epigenetic mechanism: Ten-eleven translocation methylcytosine dioxygenase (TET)-mediated DNA hydroxymethylation

TET-mediated DNA hydroxymethylation is a novel epigenetic mechanism that is involved in the DNA demethylation pathway, and therefore, a critical enzyme involved in shaping the epigenome and regulating gene expression. The aberrant activities of TET proteins and their 5hmC products are also implicated in many disease states, and have the potential to be used as biomarkers for disease development.

1.3.1 Role of TET in gene regulation

DNA methylation has been the most widely studied epigenetic mechanism; however, the counterpart to DNA methylation, DNA demethylation mechanisms remains largely unknown. There are two proposed DNA demethylation pathways: passive and active. Passive DNA demethylation is simply the loss of DNA methylation during the cell division process, either through a mistake or aberrant maintenance methylation by DNMTs. Regardless of the pathway, omitting the methylation mark results in the passive removal or loss of DNA methylation through successive cell divisions. During zygote formation, there is rapid demethylation during the zygote cleavage and rapid methylation after implantation, independent of cell division, which indicates that active DNA demethylation pathways are responsible (47, 48). The discovery of ten-eleven translocation (TET) proteins brought incredible interest to the 5hmC-mediated DNA demethylation pathways, and the biological role of 5hmC in development (49). TET enzymes are responsible for catalyzing the oxidation of 5mC to 5hmC, and the iterative oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) DNA

derivatives, believed to be the intermediates in the DNA demethylation pathways (Figure 1) (20).

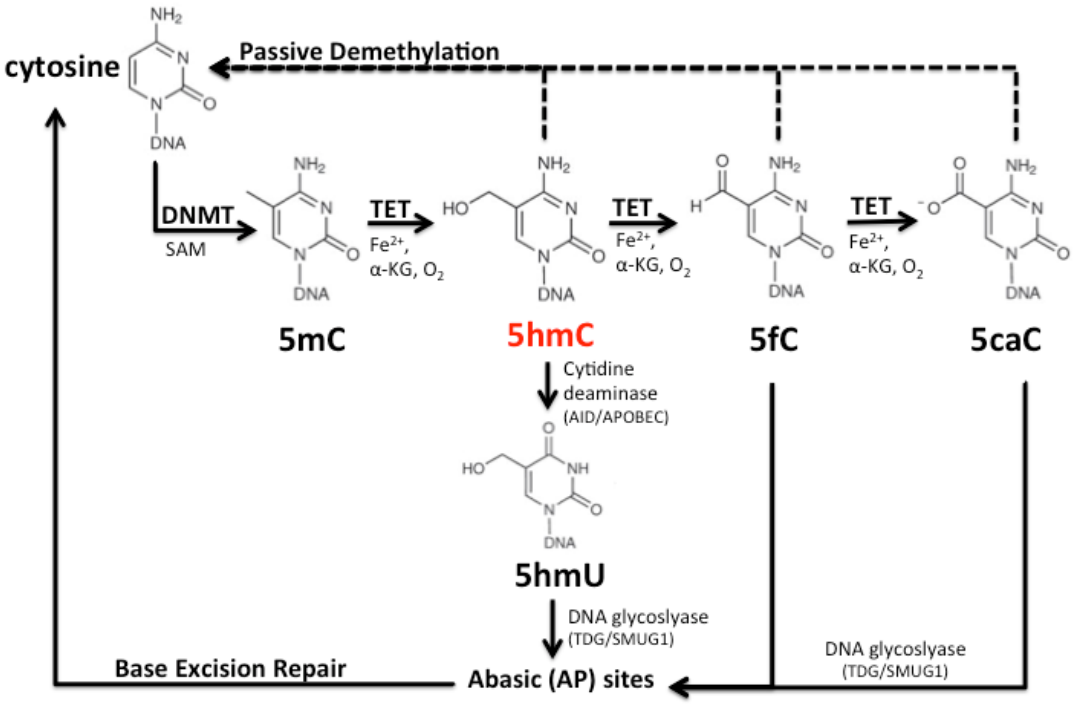

Figure 1. Chemistry of DNA methylation and hydroxymethylation. 5-Methylcytosine (5mC) is produced from the addition of S-adenosylmethionine (SAM) onto the 5-carbon of cytosine by DNA methyltransferase (DNMT). Ten-eleven translocation (TET) proteins then catalyze the iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formlycytosine (5fC), and 5-carboxycytosine (5caC), with the required cofactors: alpha-ketoglutarate (alpha-KG), iron (Fe^{2+}), and oxygen. 5hmC, 5fC, or 5caC could act as an intermediate in both the passive and active DNA demethylation pathways. The active DNA demethylation pathway involves cytidine deaminases (AID/APOBEC) and DNA glycosylase enzymes (TDG/SMUG1) to generate AP site intermediates for base excision repair pathways, which recover an unmodified cytosine. (Adapted from Dao et al., Curr Environ Health Rep, 2014)

TET expression levels vary between the cells and organs. TET1 and TET2 are highly expressed in mouse embryonic stem cells, while TET3 is seen predominantly in oocytes and one-cell zygotes (47). TET1 and TET3 are expressed in several adult tissues, but most abundant in the brain, while TET2 is enriched in hematopoietic cells (50). All of the TET proteins have a catalytic domain that contains both a cysteine-rich region and 2-

oxoglutarate-Fe(II) dioxygenase activity (51-53). In mammals, TET proteins (TET1, TET2, and TET3) catalyze the transfer of a hydroxyl group to 5mC (52-54) to form 5hmC/5fC/5caC. TET1 can oxidize both fully and hemi-methylated DNA, and does not require CpG dinucleotides (47). In the N-terminal, the CXXC domain of the TET proteins (TET1 and TET3 only) is a DNA binding domain, with a high affinity for clustered unmethylated DNA (47, 53). Subsequent genome-wide mapping of mouse and human embryonic stem cells has determined that the CXXC domain of TET1 overlaps with the unmethylated DNA generally located in the CPG islands (CGIs) of promoter regions (47), signaling many chromatin-associated proteins. TET mediated DNA hydroxymethylation can regulate gene transcription through the non-enzymatic and enzymatic pathways involved in the DNA demethylation pathway (20).

Non-enzymatic regulation refers to the role of 5hmC to activate gene expression, without any enzymatic activity, by destabilizing the DNA structure to allow the transcriptional machinery to access the transcriptional start site (50). The degree of 5hmC content can reduce the methylation binding domain (MBD) interactions, to reorganize the chromatin structure and reverse the repressive effects of 5mC on transcriptional regulation (20).

Enzymatic regulation refers to the role of 5hmC as the “sixth base” in DNA, and the critical intermediate in the active DNA demethylation pathway (55). In the DNA demethylation pathway, 5hmC is recognized by activation-induced cytidine deaminase (AID/APOBEC), which mediates the deamination of 5hmC to 5-hydroxymethlyuracil (5hmU), generating an abasic site. This is recognized and subsequently removed by the DNA glycosylases (SMUG1, single-strand-selective monofunctional uracil-DNA

glycosylase 1) and TDG (thymine DNA glycosylase), and the base excision repair machinery, to restore an unmodified cytosine (Figure 1) (47, 51).

In summary, there are three main mechanisms that 5hmC modifications are generally considered to transcriptionally activate: 1) 5hmC replaces the repressive 5mC modifications to remove the MBD proteins and allow transcription factor binding, 2) 5hmC is involved in the DNA demethylation pathway to recover an unmodified cytosine, and 3) 5hmC is suggested to recruit proteins to induce active gene transcription (20). Thus, TET-mediated DNA hydroxymethylation plays critical roles in altering the epigenome and gene regulation, which can result in both biological and disease consequences.

1.3.2 Role of TET and DNA hydroxymethylation in disease development

Cytosine modifications, such as 5mC and 5hmC, are epigenetic mechanisms that enable genes to respond to external environmental cues. Environmental exposure has been shown to disrupt epigenetic mechanisms and interfere with the 5mC and 5hmC profiles in many diseases. TET proteins are also apt to environmental perturbations; therefore, researchers have been investigating 5hmC content as a novel epigenetic marker to understand the link between epigenetic signatures and disease states.

With regard to cancer, in tissues from the human lung, brain, kidney, liver, skin, and small intestine, the levels of 5hmC are remarkably depleted in the malignant tissues compared to the benign tissues (56-58). One explanation is that tumor formation involves rapid cell proliferation, which could lead to the passive loss of 5hmC (58). In addition, hepatocellular carcinoma samples showed that lower levels of 5hmC were associated

with decreased overall survival in hepatocellular carcinoma (57). Therefore, there is evidence for the use of 5hmC levels as novel biomarkers of disease development. The immunohistochemical (IHC) detection of 5hmC in human glioblastomas revealed that 5hmC decreased significantly with the progression of the tumor grade, and that the reduction in 5hmC became a significant prognostic indicator of decreased life expectancy in fetal and adult glioblastomas (56).

In bone marrow samples from mixed lineage leukemia (MLL)-rearranged acute myeloid leukemia patients (TET1 is a fusion partner of the MLL gene), TET1 was overexpressed (59). Additionally, in myelodysplastic syndrome (MDS), TET2 and 5hmC expression appear to be necessary for the normal differentiation of hematopoietic stem cells (60, 61). TET2 mutations in bone marrow mononuclear cells were associated with ~16% of MDS patients, and the levels of 5hmC were significantly lower in MDS patients with mutant TET2 (62). After risk stratifying the 5hmC patients, it was found that higher 5hmC levels had increased MDS survival rates when compared to those with low 5hmC levels (62).

Increasing evidence has implicated the role of epigenetic modifications in neuropsychiatric disorders, especially since TET1 and TET3 are highly expressed in brain tissues. Using post-mortem brain cortex tissue from psychotic (bipolar disorder, schizophrenia) and depressed patients to examine the TET1 mRNA and protein expression, it was shown that TET1 expression levels were increased in both the psychotic and depressed patients when compared to the controls (63). In another study, the global 5hmC levels were significantly increased in psychotic patients when compared

to the controls (64). Altogether, these studies demonstrate that TET-mediated DNA hydroxymethylation generates unique epigenetic signatures that are associated with disease phenotypes.

Epigenetic mechanisms provide a useful relationship to record environmental exposures that may interfere with disease mechanisms. Most importantly, epigenetic mechanisms can be reversed or modified to alter disease states, which provides tremendous therapeutic potential for epigenetic therapies. Asthma is a chronic inflammatory disease, coupled with airway remodeling, that affects many people worldwide, and there are no successful therapies, only palliative treatments. Although asthma research has focused heavily on the immunological aspects of asthma pathogenesis, the success rate of new immunotherapy based biologics has varied in patients. Therefore, an investigation into the alternative mechanisms (other than immunological responses) in the pathogenesis of asthma can provide useful insights into the disease progression.

Currently, the exploration of the epigenetic mechanisms underlying asthma pathogenesis is predicted to provide novel insight into the diagnosis and treatment of asthma. This thesis research investigates the role of DNA hydroxymethylation, a novel epigenetic mechanism, in the pathogenesis of asthma. In Chapter 2, we aim to determine whether the experimental asthma model of Tet1-deficient mice exposed to house dust mite was protected from allergen-induced AHR and aberrant lung DNA methylation when compared to allergen-exposed wild-type mice. Furthermore, we investigate whether TET1-mediated hydroxymethylation plays a role in modulating the ASM cells of asthmatics. In Chapter 3, we explore the underlying mechanisms of TET1 activation in

human ASM cells, and aim to determine if the modulation of the levels of oxidative stress is able to alter the epigenetic states and ASM cell phenotypes (cell proliferation, contraction, and collagen synthesis).

CHAPTER 2

THE ROLE OF TET1 UPON HDM-DRIVEN AHR IN AN EXPERIMENTAL ASTHMA MODEL

ABSTRACT

Asthma, a chronic inflammatory disease of the airways that leads to airway narrowing, has been recognized as having both genetic and environmental predispositions. Recent research has focused on the role of environmental perturbations on the disease pathogenesis of asthma through epigenetic modifications. Previously, in a mouse model, we demonstrated that house dust mite (HDM) allergen exposure induced airway hyper-responsiveness (AHR), and inflammatory immune responses associated with DNA methylation changes in the mouse lung. Here, we investigated the effects of the ten-eleven translocation 1 (TET1) protein, which catalyzes the oxidation of 5-methylcytosine into 5-hydroxymethylcytosince, upon HDM-induced AHR in a *Tet1*$^{+/-}$ mouse model. We found that the Tet1 deficient mice were protected from HDM-induced AHR, and had decreased airway inflammation, collagen deposition, and mucus formation. In both the mouse lung and tracheal derived airway smooth muscle cells (ASMCs), we observed attenuation of the AHR-related gene expression and gene-specific methylation changes, with the loss of Tet1. Furthermore, we investigated the role of TET1 in human ASMCs from non-asthmatic and asthmatic patients. The knockdown of *TET1* by small-interfering RNA in asthmatic ASMCs reduced the cell proliferation and gene expression of PCNA, SMA, CAMK2D, ADAM33, and TGFβ2. Herein, we demonstrated that TET1-mediated epigenetic modifications are associated with changes in AHR-related phenotypes in both an experimental asthma model and human ASMCs from asthmatic patients. Our findings could provide insight into the epigenetic role of TET1 in asthma pathogenesis, and provide opportunities for novel strategies for disease prevention and intervention.

INTRODUCTION

Asthma has become a major public health concern in developed countries, with the rates of diagnosis increasingly common and contributing to the third leading cause of hospitalization among children in the United Sates (65). Asthma is characterized by the exaggerated narrowing of the airways upon contact with stimuli, resulting in airway hyper-responsiveness (AHR) (65). Furthermore, asthma is a complex chronic inflammatory disease of the airways, involving increased airway smooth muscle mass, increased collagen deposition and increased fibrosis. These structural changes are referred to as airway remodeling (30, 66). Across the population, the rising rates of asthma susceptibility cannot be explained by genetic determinants alone (30, 65, 67). For individuals, the association between the relative risk of asthma and genetic determinants is uncertain, due to the unique environmental exposures every individual encounters (67). Therefore, the prevalence of asthma is proposed to be associated with both genetic and environmental factors, and epidemiological evidence suggests increased asthmatic risks or symptoms with a wide range of environmental exposures. For example, exposure to phthalates was associated with an increased asthma risk in children (43). Among phthalate-contaminated children, increased phthalate urinary metabolite mono(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP) was associated with decreased *TNF-alpha* DNA methylation and increased *TNF-alpha* phthalate urinary metabolite (43). In another study, inner city asthmatic subjects showed many differentially methylated regions, compared to non-asthmatic subjects, including the hypomethylation of the *IL13* and *IL4* immune genes, measured in the circulating peripheral blood mononuclear cells (PBMCs) (24). Additionally, in asthmatic subjects, ambient polycyclic aromatic hydrocarbon

(PAH) exposure from combustion sources was associated with increased *FOXP3* DNA methylation, and decreased gene expression and protein levels of FOXP3 in T-regulatory immune cells (8). Furthermore, maternal exposure to PAHs was shown to be associated with increased cord blood *ACSL3* methylation and the risk of childhood asthma (68).

Epigenetic mechanisms are responsible for maintaining the diverse cellular types and functions from the identical genetic code in each cell. In general, there are three basic mechanisms of epigenetic regulation, 1) DNA methylation, 2) histone modifications, and 3) microRNAs; they all work dynamically to regulate gene transcription. DNA methylation, the most widely studied epigenetic modification, involves the covalent addition of a methyl group from S-adnenosyl methionine (SAM) to the C5 position of cytosine in CpG dinucleotides, which results in a bulky methyl group that blocks the transcription factor or transcriptional machinery binding to repress gene transcription (2, 20). Therefore, alterations in the epigenetic mechanisms can disrupt the epigenome (epigenetic genome) and dysregulate gene transcription, which may lead to the aberrant cell phenotypes implicated in disease pathogenesis. In asthma, aberrant DNA demethylation and chromatin modification are widely studied and associated with increased Th2 gene expression and chronic inflammation (42, 69-71), this skewed Th2 phenotype is a hallmark of asthma pathogenesis. Nevertheless, few studies have examined epigenetic changes in lung tissue directly.

Previously, we demonstrated that an allergen challenge with HDM induced an allergic asthma phenotype in mice that was associated with epigenetic changes (38, 45).

HDM is a ubiquitous environmental allergen commonly found in carpets and mattresses inside the home (35). In animal models, an HDM-challenge induced an allergic asthma phenotype, increasing airway hyper-responsiveness (AHR), airway smooth muscle (ASM) hyperplasia, and allergic inflammation (33, 35, 37, 38). We demonstrated that global increases in DNA hydroxymethylation were coupled with decreased gene specific DNA methylation in both lung tissues and tracheal ASM cells isolated from HDM-exposed mice. Furthermore, the expression levels of differentially methylated genes were associated with aberrant ASM cell phenotypes. Upon examining a broad range of epigenetic components that could be responsible for the DNA methylation changes from HDM exposure, we identified the increased expression of the genes involved in the DNA demethylation pathways: TET1 (ten-eleven translocation protein or 5-methylcytosine dioxygenase 1), AID (activation-induced cytidine deaminase), and SMUG1 (single-strand-selective monofunctional uracil-DNA glycosylase 1) with HDM-exposure (45). These results indicated that alterations in DNA hydroxymethylation (a possible intermediate in the DNA demethylation pathway) may contribute to the increased AHR in mice.

DNA hydroxymethylation is mediated by a family of TET proteins (TET1, TET2, and TET3), which are made up of 2-oxoglutarate-Fe(II)dioxygenase that catalyzes the addition of a hydroxyl group onto methylated DNA, converting 5mC (5-methylcytosine) into 5hmC (5-hydoxymethylcytosine) (20, 72). In addition, 5hmC is a demethylation intermediate that can be iteratively oxidized by TET proteins to form the additional intermediates 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC) (20, 72). 5hmC, and

possibly its intermediates, can undergo deamination (AID/APOBEC enzymes) followed by base excision repair (TDG/SMUG1) to remove the DNA methylation moiety and generate cytosine (60). Furthermore, 5hmC, and possibly its intermediates, can passively remove DNA methylation through the inability to replicate the 5mC modification during DNA replication (60). Therefore, TET proteins are required to catalyze 5mC into 5hmC, a key epigenetic modifier in the DNA demethylation pathway. TET-mediated DNA hydroxymethylation may play critical roles in regulating DNA methylation changes in the epigenome to alter gene transcription profiles, resulting in aberrant phenotypes. TET proteins can remove repressive DNA methylation markers, and therefore, permit transcription factor and RNA polymerase binding to increase gene transcription. Consequently, the role of TET proteins in DNA hydroxymethylation has been implicated as a novel epigenetic mechanism and biomarker of disease (2, 20). This suggests that understanding the TET-mediated DNA hydroxymethylation in disease development may provide unique therapeutic opportunities for curing disease.

There are few mouse models to investigate the effects of the Tet family of proteins *in vivo*. Mice with the whole body knockout of Tet2 are fertile and viable, but during development approximately 34% of the *Tet2*$^{+/-}$ and 8% of the *Tet2*$^{-/-}$ mice died of lethal myeloid malignancies (73). In addition, conditional Tet2 mutant models displayed increased susceptibility to chronic myelomonocytic leukemia and increased mortality during development (74). Clinical findings indicate that among human hematopoietic malignancies and diseases, there are prevalent mutations and deficiencies in the Tet2 protein (74-76), thereby supporting the biological relevance of the Tet2 mouse model

findings. Tet3 proteins are essential for embryonic development; therefore, the homozygous mutation of Tet3 leads to neonatal lethality (77). The double knockout mouse model of Tet1 and Tet2 proteins showed increased perinatal lethality when compared to a single Tet1 or Tet2 knockout (78), further indicating that Tet1 and Tet2 proteins are required for postnatal development. We employed mixed background C57BL/6J x 129S4/SvJae *Tet1*$^{+/-}$ mice that were heterozygous for the targeted Tet1 mutation at exon 4, which contained the catalytic domain (79). Using the same *Tet1*$^{+/-}$ or *Tet1*$^{-/-}$ mouse model, researchers demonstrated that the ablation of Tet1 in mouse brains caused impaired memory extinction and significantly down-regulated the neuronally related genes in the hippocampus and cortex, although these mice displayed similar gross morphology and behavioral assessments when compared to those of *Tet1*$^{+/+}$ (80). Therefore, the use of the genetic knockout model of Tet1 may allow us to investigate the physiological effects of Tet1 during development or disease pathogenesis.

In this study, we aimed to examine the role of Tet1 upon HDM-induced AHR. We exposed both Tet1 wild-type (*Tet1*$^{+/+}$) and heterozygous (*Tet1*$^{+/-}$) mice to HDM, and examined the phenotypes of AHR and DNA methylation changes in both the lungs and isolated tracheal ASM cells. In addition, we determined whether the knockdown of TET1 in human ASMCs could modulate the aberrant ASM cell phenotypes seen in asthmatics. Our findings may provide insight into the novel epigenetic mechanisms that regulate ASM phenotypes that could contribute to AHR.

METHODS

Animal Design

Mice housing

All of the experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD) (MO14H241). For the generation of the heterozygous *Tet1*$^{+/-}$ knockout, adult *Tet1*$^{+/-}$ female mice from the C57BL/6J/129S4/SvJae (B6/SV129) strain were bred to *Tet1*$^{+/+}$ control wild-type male mice from strain B6/SV129. Both the heterozygous and wild-type control mice were obtained from Jackson Laboratories (Bar Harbor, ME). The litters were weaned at 21 days of age, and genotyped before they were used for the proposed experiments. All of the mice were housed under controlled lighting (12 h light and 12 h dark) and temperature (21–22°C) conditions. The mice were housed in polysulfone-ventilated cages (Technoplast, Exton, PA), and provided with Harlan's Teklad Global 18% Protein Extruded Rodent Diet 2018SX and fresh water ad libitum.

Tet1$^{+/-}$ *mice and genotyping*

DNA was extracted from tail snips (<5 mm) that were collected from mouse pups that were less than 21 days old, using the AccuStart II Mouse Genotyping Kit (Quanta Biosciences, Gaithersburg, MD). The genotypes were analyzed from the DNA extract with endpoint PCR using the primers provided by Jackson Laboratories: TET1 mutant primer (5' AAC TGA TTC CCT TCG TGC AG 3'), wild-type forward primer (5' TCA GGG AGC TCA TGG AGA CTA 3'), and common reverse primer (5' TTA AAG CAT GGG TGG GAG TC 3'). The PCR cycling conditions consisted of 94°C for 90 seconds, followed by 35 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 45 seconds, with a final stage of 72°C for 2 minutes before holding at 4°C. After PCR

cycling, the PCR products were run on a 2% ethidium bromide (0.5 μg/mL) stained agarose gel, alongside a DNA ladder (New England BioLabs, Ipswich, MA), and visualized under UV light for the genotype analysis.

Exposures to house allergens

The house dust mite (HDM) extract (*Dermatophagoides pteronyssinus*) was purchased from Greer (Lenoir, NC) in a lyophilized form, before being diluted with phosphate buffered saline (PBS), and aliquoted appropriately so that all of the treatments in a single experiment used the same batch of HDM extract. Male mice, at 6 to 8 weeks of age, were sensitized on day 0 (treatment day) with 50 μg of HDM (diluted in 200 μL PBS) or PBS intraperitoneally (i.p.). Two weeks later (day 14), the mice were challenged with 50 μg of HDM (diluted in 50 μL PBS) or PBS (intratracheally, i.t.). For the acute HDM-induced airway hyper-responsiveness (AHR) model, two more HDM/PBS challenges were given to the mice i.t. on day 18 and day 21, for a total of 3 HDM or PBS challenges, before the AHR endpoints were measured on day 23.

Pulmonary Function Test (PFT) for AHR Measurement

Two days following the final HDM or PBS challenge, the mice were anesthetized i.p. with pharmaceutical grade ketamine and xylazine. After approximately 15 minutes, a 20-gauge IV catheter was placed in the trachea and secured with a suture, and the mice were maintained on a mechanical ventilator delivering a constant inspiratory flow of air. The mice were administrated with increasing doses of aerosolized methacholine chloride (MCh) (Sigma-Aldrich, St. Louis, MO) (0.1, 0.3, 1, 3, 10, and 30 mg/mL) as described in a previous study (81), while being maintained on the ventilator. The MCh challenge was followed by an airway resistance measurement using a flexiVent (SCIREQ, Montreal,

Canada). The AHR was assessed as the change in pulmonary resistance (R cm/H_2O/mL/s) compared to the baseline after the MCh challenge.

Cardiac Puncture and Collection of Blood and Serum

Following the MCh challenge, a cardiac puncture was performed with a 1 mL syringe needle filled with 10 µL of ethylenediaminetetraacetic acid (EDTA) (Quality Biological, Gaithersburg, MD). The blood sample was incubated with the EDTA at room temperature for 20 minutes before being layered on top of the Ficol-Paque (GE Healthcare, Uppsala, Sweden) reagent. The sample was then centrifuged at room temperature for 30 minutes at 5000 rpm to collect both the serum and lymphocytes.

Enzyme-linked Immunosorbent Assay (ELISA) to measure the HDM-IgE in the Serum

For the HDM-specific ELISA's, HDM was diluted to 100 µg/ml and plated in 96 well-plates overnight at 4°C. After washing, the plate was incubated with 1xPBS (with 10% fetal bovine serum, FBS) for 2 hours at room temperature, before the diluted (in 1xPBS with 10% FBS) serum samples were added and incubated overnight at 4°C. After washing, the diluted detection antibody (2 µg/ml) for the IgE (BioLegend, San Diego, CA) was added to the plate, and the plate was incubated at room temperature for 45 minutes. Avidin peroxidase (Sigma-Aldrich, St. Louis, MO) was added to the plate, which was incubated for 30 minutes at room temperature before color development with 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (ThermoScientific, Rockford, IL). The color development was read at 405/595 nm.

Collection of Bronchial alveolar lavage fluid (BALF) and Cell Counts

Following cardiac puncture, 1 mL of ice cold 1x Phosphate-buffered Saline (PBS) (supplemented with Pierce Protease Inhibitor Mini Tablets, EDTA-free; ThermoScientific, Rockford, IL) was slowly injected into the trachea using a 1 mL syringe without a needle, through the same secured trachea catheter for the airway resistance measurement. The bronchoalveolar lavage (BAL) cells collected in the recovered PBS from the airways were centrifuged, and further washed with the 1xPBS mixture. The BAL cells were resuspended in 1xPBS with protease inhibitor, and then stained with Turk's solution (Ricca Chemical, Pocomoke City, MD), before being counted using a hemocytometer (Paul Marienfeld, Lauda-Koenigshofen, Germany). Subsequently, 0.1 mL of the BAL cells was added to cytology funnels (Shandon™ disposable cytofunnels, ThermoScientific, Rockford, IL) clamped to Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburg, PA), and loaded into the Cytospin centrifuge (Shandon™ Cytospin II Cytocentrifuge, ThermoScientific, Rockford, IL) at 600 rpm for 5 minutes and dried overnight. After drying, Cytoseal™ 60 (ThermoScientific, Rockford, IL) adhesive was used to secure a glass cover slip onto the slides. Differential cell counts were obtained under light microscopy after staining with Hema-3 stain (Fisher Scientific, Pittsburg, PA), with a total of 400 cells (neutrophils, eosinophils, lymphocytes, and monocytes) counted per stained slide.

Assessment of Airway Inflammation

After the AHR measurement, the chest wall and diaphragm were exposed, and the right mainstem bronchus was tied off with suture. The right lung was removed, snap frozen in liquid nitrogen, and stored at –80°C for DNA, RNA, and protein analyses. The left lobe was inflated with zinc-buffered formalin (Z-Fix, 174; Anatech, Battle Creek, MI) at a

constant pressure of 30 cmH_2O. The trachea was tied, and the lungs were excised and submerged in Z-fix for at least 24 hours. Then, the lungs were processed, embedded in paraffin using a routine histological procedure, and cut into 5 μm sections. The sections were subsequently stained with either hematoxylin and eosin (H&E) to assess the general lung morphology and inflammatory cells, Masson's trichrome staining (MTS; American Mastertech Scientific, Inc.) to assess the subepithelial fibrosis and collagen deposition, or periodic acid-Schiff (PAS; Sigma-Aldrich) to assess the presence of the goblet cells in the epithelium.

Tissue Collection

After the AHR assessment in the mice, the tissues (lung, spleen, brain, and trachea) were harvested and processed for the gene expression analysis and immunohistochemical study. The right lung was excised for RNA, DNA, and nuclear protein isolation. The trachea was collected to isolate airway smooth muscle cells (ASMCs) as previously described (82).

Isolation of Tracheal ASM Cells from Mice

Immediately following collection, the tracheal tissue was placed in RPMI 1640 media (Corning Cellgro, Manassas, VA) supplemented with 1% penicillin-streptomycin (Corning Cellgro, Manassas, VA). The tracheal tissue was rinsed in RPMI 1640 media, and the surrounding tissue was dissected away before the tracheal segment was split longitudinally and placed intima side down in a 100 mm dish (with a grid to fix the position). Then, 5 ml of the RPMI 1640 media containing 20% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate was added to the 100 mm dish. After 3 days of incubation at 37°C, the media was changed to RPMI 1640 containing 10% FBS, 1%

penicillin-streptomycin, and 1% sodium pyruvate. The tracheal tissue was removed when the outgrowing cells became locally confluent. After approximately 7-10 days, the mouse ASMCs were harvested and the cell purity assessed by immunocytochemistry using antibodies against cytokeratin, vimentin, tropomyosin, and smooth muscle actin (Santa Cruz Biotechnology, CA).

DNA Isolation

The DNA isolation was performed using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA), following the instructions from the manufacturer. In brief, 50-100 mg of tissue were incubated overnight with digestion solution at 56°C to completely lyse the tissue before proceeding with the DNA binding, washing, and elution. For mammalian cells grown in the monolayer, the cells were collected from the culture dish and centrifuged at 2000 rpm for 3 minutes. The cells were resuspended in 200 μL of 1xPBS, and the DNA was isolated using the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The concentration of the isolated DNA was determined by using a Take3 Micro-Volume Plate (BioTeK, Winooski, VT) and quantified using the Gen5 analysis software in the BioTek Epoch Spectrometer system (BioTek, Winooski, VT). For the tissues, the integrity of the isolated DNA was assessed by running the DNA on an ethidium bromide (0.5 μg/mL) stained agarose gel. The DNA was visualized under UV light.

Global 5mC and 5hmC Levels in DNA

Two hundred ng of genomic DNA from the mouse lung tissues or cell cultures was used to measure the levels of 5-methyl-cytosine (5mC) and 5-hydroxymethyl-cytosine (5hmC) in the DNA using the 5mC DNA ELISA Kit and Quest 5hmC™ DNA ELISA Kit (Zymo

Research, Irvine, CA). The percent of 5mC and 5hmC of the samples was quantified, compared to the standard curve provided in the kit.

TET Activity

The nuclear protein extracts were isolated from the cells using the EpiQuick Nuclear Extraction Kit (Epigentek, Farmingdale, NY). To collect the cytosolic protein, the cells were lysed in RIPA buffer (Cell Signaling, Danvers, MA) in the presence of a protease inhibitor cocktail (Invitrogen, Carlsbad, CA). The proteins were quantified for the total protein levels (Pierce BCA Protein Assay Kit; ThermoScientific, Rockford, IL), and 2 to 6 μg of the total nuclear or cytosolic protein were used to measure the TET activity (Epigentek, Farmingdale, NY). The TET activity was assayed using the Epigenase 5mC Hydroxylase TET Activity/Inhibition Kit (Epigentek, Farmingdale, NY), in which the TET substrate was added to all of the wells and incubated at 37°C for 90 minutes. Then, 2-6 μg of the total nuclear or cytosolic protein at a standard curve were incubated with the TET substrate at 37°C for 90 minutes to convert the TET substrate into the 5hmC product. The capture antibody was added to recognize the TET-converted products, and the detection antibody was incubated. Finally, for the signal detection, the colorimetric conversion of the developer solution was measured at 405/655 nm, and the TET activity was calculated compared to the standards.

RNA Isolation

For the tissues, 50-100 mg of tissue was added to pre-filled microtubes with 1.5 mm zirconium beads (Benchmark Scientific, Edison, NJ) with 1 mL of TRIzol (Invitrogen, Carlsbad, CA). The microtubes were inserted into the BeadBug Microtube Homogenizer (Benchmark Scientific, Edison, NJ) and vortexed at 4000 rpm for 30 seconds.

Afterwards, RNA was isolated following the manufacturer's protocol for TRIzol. For the mammalian cells grown in the monolayer, cells were collected from the culture dish and centrifuged at 2000 rpm for 2 minutes. TRIzol was added directly to the cell pellet, and the RNA was isolated from the mammalian cell culture by the manufacturer's protocol for TRIzol. The concentration of the total RNA was determined using a Take3 Micro-Volume Plate (BioTeK, Winooski, VT) and quantified with the Gen5 analysis software in the BioTek Epoch Spectrometer system (BioTeK, Winooski, VT). For the tissues, the integrity of the isolated RNA was assessed by running the RNA on an ethidium bromide (0.5 μg/mL) stained agarose gel. The RNA was visualized under UV light.

Real-time Reverse Transcriptase PCR (RTPCR)

The total RNA (1 μg) was isolated from the lung tissues, isolated mouse ASMCs, and human ASMCs, and reverse transcribed with iScript Reverse Transcriptase (BIO-RAD, Hercules, CA). The mRNA levels of the genes were quantified by TaqMan-based or SYBR Green-based real-time PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of the transcripts normalized to *RPL19*. The primers are listed in Table 1 and described in previous studies (14, 15).

Methylation Specific PCR (MSPCR)

As a starting material, 200 ng of genomic DNA was used for bisulfite treatment using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA). The bisulfite treated DNA (20 ng) was amplified with methylation specific primers using conventional PCR (GoTaq Green Master Mix; Promega, Madison, WI). The PCR products were loaded onto a 2% agarose gel for electrophoresis, and the levels of the PCR product were densitometrically analyzed (GelQuantNET) and standardized to β-actin. The control DNAs (fully

methylated and fully unmethylated; Zymo Research, Irvine, CA) were mixed in various concentrations and served as quantification standards (0, 25, 50, 75, and 100% methylated DNA) when determining the methylation ratio of the samples from the PCR. The primers were listed in Table 1 and described in previous studies (14, 15).

Cell Culture

Human ASM cells from non-asthmatics or asthmatics were either purchased from a commercial vendor (Lonza, Gaithersburg, MD) or obtained from deceased donors (the donor demographics are listed in Table 6) from the National Disease Research Interchange (Philadelphia, PA), as previously described (83). The cells were received at Passage 3 and maintained in DMEM F-12 medium (Corning Cellgro, Manassas, VA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin solution (Corning Cellgro, Manassas, VA), and 1% L-glutamine (Corning Cellgro, Manassas, VA). The cells were not used once they reached Passage 12.

siRNA Transfection

The human ASMs were grown to 70% confluence at the time of transfection. The cells were incubated with 50 nM si*TET1*, si*TET2*, or non-targeting control (siCTL) oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as the carrier for 3 days. The sequences were as follows: si*TET*1 RNA oligonucleotides (Dharmacon #J-014635-23-0010) target sequence GAGAAUAGGUAUGGUCAAA, siTET2 RNA oligos (Dharmacon #J-013776-25-005) target sequence CAGCAAAGGUACUUGAUAC, and siCTL RNA oligos which is a pool of negative control non-targeted RNA oligos (Dharmacon #D-001810-10-05) target sequences UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, and

UGGUUUACAUGUUUUCCUA. After 3 days of incubation, the cells were harvested for the RNA (for qPCR), DNA (for 5mC and 5hmC measurements), nuclear extract (for TET activity), and 5-bromo-2-deoxyuridine (BrdU assay).

5-Bromo-2-Deoxyuridine (BrdU) Assay for Cell Proliferation

The human ASMCs were incubated with a 5-bromo-2'-deoxyuridine (BrdU) solution for six hours, according to the BrdU Cell Proliferation Assay Kit (Cell Signaling, Beverly, MA). The BrdU analog incorporation into the DNA was evaluated using the color formation measured at an absorbance of 450 nm.

Statistical Analysis

The results were expressed as the mean ± standard error mean (SEM), and Student's t-test was used to analyze the differences in the ASM characteristics between the non-asthmatic and asthmatic ASM cells. For the group comparisons, a two-way ANOVA of multiple comparisons with Bonferroni's or Tukey's corrected tests were applied to determine if data between the treatment groups was statistically significant. The details of the statistical analysis are described in each data figure and table. All of the data were analyzed and plotted with Prism6 (GraphPad, La Jolla, CA).

Study Approval

All of the experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Baltimore, MD) (MO14H241). The human ASMCs from the non-asthmatics and asthmatics were either purchased from a commercial vendor (Lonza, Gaithersburg, MD) or obtained from deceased donors in accordance with the Institutional Review Board at the University of Pennsylvania and Johns Hopkins University.

Table 1. Sequence of primers used for qPCR and MSPCR.

Primers for qPCR for mouse genes.

Primer Name	Sequence (5' to 3')
mAkt1s1-F	GGC GGG GTG CTG GAA AAG CG
mAkt1s1-R	CCA CGA CGG CTT CCC ACA GT
mBmp3-F	CAG CCG CAG GAA CTC CTC AAA
mBmp3-R	CAA CGT AGA AAT ACA GTG TGG CTG
mCamk2d	TaqMan assay_Mm00499266_m1
mCartpt-F	GCG CTA TGT TGC AGA TCG AAG
mCartpt-R	AGC GTC ACA CAT GGG GAC TTG
mCcnd1-F	ACC TGG GCA GCC CCA ACA AC
mCcnd1-R	GGA GGC AGT CCG GGT CAC ACT
mCol1a1-F	TTG GGT CCC TCG ACT CCT AC
mCol1a1-R	TGA CTG TCC CAC GTA AGC AC
mCol3a1-F	GAG GGC CAT AGC TGA ACT GA
mCol3a1-R	TGC AGA GTT AAC AAC AGT CAG C
mFoxp3-F	ACC AAG GTG AGC GAG TGT C
mFoxp3-R	TTT CTT CTG TCT GGA GTG GCT G
mll4-F	GCA ACG AAG AAC ACC ACA GAG
mll4-R	GCA TCG AAA AGC CCG AAA GA
mll5-F	CGT GGG GGT ACT GTG GAA AT
mll5-R	GCC ACA CTT CTC TTT TTG GCG
mll6ra-F	GAT GCC TTG CGA GGA GTG AA
mll6ra-R	CTG GGC TCT GCT ATC CAA GG
mll10-F	CAT TTG AAT TCC CTG GGT GAG AAG
mll10-R	GCC TTG TAG ACA CCT TGG TCT TGG
mll13-F	AGC TGA GCA ACA TCA CAC AAG ACC
mll13-R	TGG GCT ACT TCG ATT TTG GTA TCG
mMuc5b-F	ATG GAG TCA CTA TAC ACT CTC TGA
mMuc5b-R	CTG TCC CAA CCC CCA AAG AC
mMlck	TaqMan assay:Mm00653039_m1
mPcna-F	CTG GGA CGT CAG CTC GGG CG
mPcna-R	TTG GAC ATG CTG GTG AGG TTC ACG
mPde4d-F	AAC AGT TGC AGC GAG AGC ACC A
mPde4d-R	TCA GAG GCA CTT TTG CTC CGA GA
mPom121l2-F	CAG CTC TGG ACA GCT AAA TGG
mPom121l2-R	GGG TCC TCT TCA GTG ACA ACA
mSlc25a12-F	AAG ACC CGG ATG CAA AAC CA
mSlc25a12-R	TGG GAT CAG ACC TCG GTA CA
mSmad3-F	CAG TGA CCT GGG GAT GGT AAT
mSmad3-R	CAG TGA CCT GGG GAT GGT AAT
mTgfb2-F	CAC CAA AGT CCT CAG CCT GT
mTgfb2-R	GCT GTT CGA TCT TGG GCG TA
mTrpm2-F	ACA AAG GGT ACG TGG ATG ACC
mTrpm2-R	GAG TGT GCA GGT TCT CTT CCA

Table 1. Sequence of primers used for qPCR and MSPCR.

Primers for MSPCR for mouse genes.

Primer Name	Sequence (5' to 3')
mMS-Akt1s1-F	TAT TTG TTT GAA TAG GGT AGT AGA AAT
mMS-Akt1s1-R	AAC GCA ATA CGC CTA CGC CGT CCC G
mMS-Bmp3-F	TAA ATA AAT TTA TTG TTT GTG ACG A
mMS-Bmp3-R	ACT AAA TAA AAA CAT CTT TTC CGA C
mMS-Cartpt-F	GCG TAG AGT TTC GTT TTC GG
mMS-Cartpt-R	GAC TTC TTA TAA CCG ATT AAA ATC GAA
mMS-Pde4d-F	CGT GGT CGT TAC GTT TTA AGC
mMS-Pde4d-R	AAA AAC GCT CTC TAC TCA CCG T
mMS-Slc25a12-F	TTA TAT TGC GCG TTG GAA TTC
mMS-Slc25a12-R	ACC TTA ACC GCC ATA CTA TAC TCG
mMS-Smad3-F	GTT TTG GTT GGT TTT GTA AGG C
mMS-Smad3-R	GAA AAT CGA AAA CAC GAC GA
mMS-Tgfb2-F	ATT TAT TTG CGG AGA GAA GGA TC
mMS-Tgfb2-R	CAA AAA CGA CAA CGA TCG AC
mMS-Trpm2-F	GGT GTT TCG AAG AGG TTG ATT C
mMS-Trpm2-R	AAT ACC TAC TCA AAA ACC GCG

Primers for qPCR for human genes.

Primer Name	Sequence (5' to 3')
hADAM33-F	ACC TAG AAT GGT GTG CCA GA
hADAM33-R	GCA CAG TGG CAG TTA TGG TTG
hCAMK2D	IDT Primetime Hs.PT.56a.27233732
hCCND1	IDT Primetime Hs.PT.56a.3857509
hCOL1A-F	CAG CCG CTT CAC CTA CAG
hCOL1A-R	TCA ATC ACT GTC TTG CCC CA
hCOL3A-F	GTT GCA CGA AAC ACA CTG GG
hCOL3A-R	AAA AGC AAA CAG GGC CAA CG
hCSF2-F	GCC CTG GGA GCA TGT GAA TG
hCSF2-R	TTT CAT TCA TCT CAG CAG CAG TG
hICAM1-F	CTT CGT GTC CTG TAT GGC CC
hICAM1-R	CAC ATT GGA GTC TGC TGG GA
hIL1B-F	GGC TGC TCT GGG ATT CTC TT
hIL1B-R	AGT CAT CCT CAT TGC CAC TGT AA
hIL4-F	TCT CAC CTC CAA CT GCT TC
hIL4-R	CTG CTC TGT GAG GCT GTT CA
hIL6-F	CAG CCC TGA GAA AGG AGA CAT
hIL6-R	GGT TCA GGT TGT TTT CTG CCA
hIL8-F	ACC GGA AGG AAC CAT CTC AC
hIL8-R	GGC AAA ACT GCA CCT TCA CAC
hMYLK2	IDT Primetime Hs.PT.56a.27638038
hPCNA-F	TCC CTT ACG CAA GTC TCA GC
hPCNA-R	AGT CTA GCT GGT TTC GGC TT

Table 1. Sequence of primers used for qPCR and MSPCR.

Primers for qPCR for human genes.

Primer Name	Sequence (5' to 3')
hSMA-F	CCG GGA CTA AGA CGG GAA TC
hSMA-R	TTG TCA CAC ACC AAG GCA GT
hSMAD2-F	GGT GGC AGG CGG GTC TA
hSMAD2-R	GCC TCT TGT ATC GAA CCT CCC
hSMAD3-F	AAC GGG CAG GAG GAG AAA TG
hSMAD3-R	ATC CAG GGA CCT GGG GAT G
hTET1-F	CGA GTT GGA AAG TTT GCC CG
hTET1-R	CAC AAG GTT TTG GTC GCT GG
hTET2-F	TGG CTG CCC TTT AGG ATT TGT
hTET2-R	GAA TGT TTG CCA GCC TCG TTC
hTGFB1-F	CGG CCT TTC CTG CTT CTC AT
hTGFB1-R	TCT TCT CCG TGG AGC TGA AG
hTGFB2-F	TTC TTC CCC TCC GAA ACT GTC
hTGFB2-R	GGT CTG TAG AAA GTG GGC GG
hTNFA-F	TGC TTG TTC CTC AGC CTC TT
hTNFA-R	CAG CTT GAG GGT TTG CTA CA
hVCAM1-F	ATG TCA ATG TTG CCC CCA GA
hVCAM1-R	TGC TCC ACA GGA TTT TCG GA
hVEGF-F	AAG GGA AAG GGG CAA AAA CG
hVEGF-R	AGG CTC CAG GGC ATT AGA CA

RESULTS

Increased Tet1 and 5hmC in HDM-exposed Mouse Lung and ASMCs

From our previous findings, the exposure to HDM in male C57BL/6J mice induced airway hyper-responsiveness (AHR) and epigenetic changes in the lung genome (14, 15). We demonstrated the increase in the 5hmC level in the HDM-exposed mice. To further elucidate the existence of 5hmC in the lung following repeated HDM exposure, we investigated the contents of the Tet-mediated oxidation products of DNA methylation (5hmC, 5fC, and 5caC). Via histological assessment of the lung sections, we detected no significant difference in the 5mC, 5fC, and 5caC levels in either the airway epithelial or the ASM cells between the saline and HDM-exposed groups (Figure 1). Strikingly, the HDM-exposed mice showed a substantial increase in the 5hmC staining in the ASM layer and the inflammatory infiltrate, but not in the airway epithelium (Figure 1). Next, we validated the increase in the 5hmC level in multiple tissue/cell types of the lungs using ELISA. The HDM-exposed mice showed a significantly increased 5hmC content in the blood (~30% increase) and the isolated tracheal mouse ASM cells (MASMCs) (~150% increase) (Figure 2A) when compared to the saline control group. We found no significant changes in the 5mC content in the blood, BALf, and MASMCs. These results suggested that while inflammatory cells likely contribute to the increased 5hmC content, they are not the major factor, suggesting that the major 5hmC changes might occur in ASM cells. These results are not surprising since 5mC and 5hmC exhibit cell-type specific expression profiles (47).

In addition to the global increases in 5hmC, the gene-specific changes in 5hmC were assayed in both the lung and ASM cells. In our previous study on methylation

profiling (MeDIP-seq), we identified that transforming growth factor beta 2 (Tgfβ2) signaling pathway was epigenetically altered by HDM exposure (45). Therefore, we examined whether the Tgfβ2 signaling genes, e.g. *Tgfβ2*, *Smad2*, and *Smad3*, showed an increase in DNA hydroxymethylation. The HDM-exposed mice showed 3-fold and 2-fold increases in the 5hmC enrichment at the lung *Tgfβ2* and *Smad2* promoters, respectively (Figure 2B). While we found no statistically significant change in the 5hmC enrichment at the *Smad3* promoter in the lung tissues of the HDM exposed mice, there was a slight increase in the 5hmC enrichment at the *Smad3* promoter ($p=0.062$) in the ASM cells. Hydroxymethylation of *Tgfβ2* was increased by 50-fold in the ASMCs of HDM-exposed mice when compared to that of the saline control. Furthermore, we found that the HDM-exposed mice showed increased enzymatic activity in the Tet proteins, in both lungs (~200% increase), and the MASMCs (~120% increase), when compared to the saline control (Figure 2C). The increase in the lung and ASMC Tet activity is in accordance with the increases in the 5hmC content shown in the lung sections (Figure 1) and MASMCs (Figure 2A). We previously reported that there was a substantial increase in the *Tet1* expression (but not in *Tet2* or *Tet3*) (15). Therefore, these findings suggested that the increase in the Tet activity might be accounted for by *Tet1* up-regulation. Taken together, these results indicate that exposure to HDM may drive the Tet-mediated DNA hydroxymethylation in both the lungs and MASMCs, which may contribute to the increased AHR.

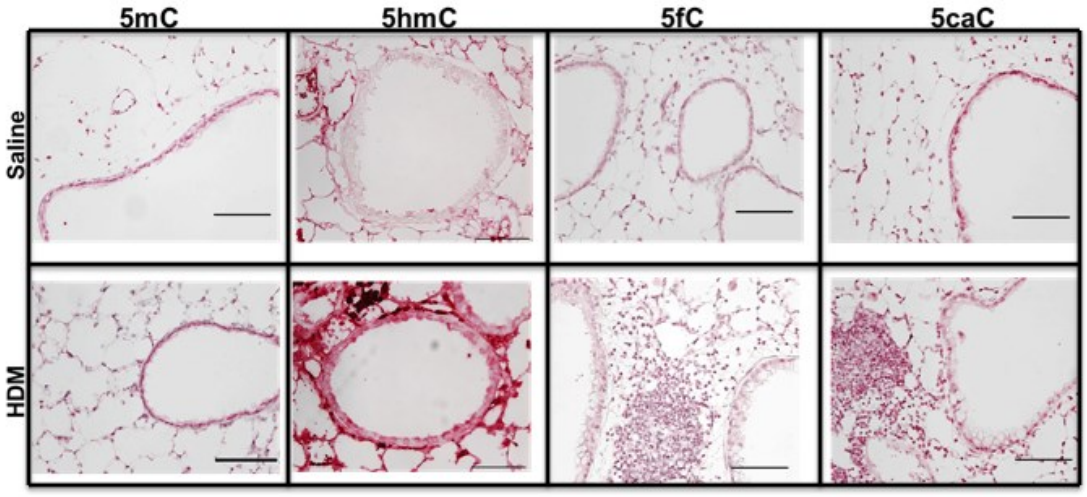

Figure 1. Significant increases in 5hmC in HDM-exposed mouse lungs. Levels of 5mC, 5hmC, 5fC, and 5caC were identified by immunohistochemical staining with the antibody specific for these 5C derivatives (scale bar=100 μm). Representative images of the lungs from each group are presented. The 5hmC levels were significantly increased after HDM exposure, especially in the cells around the airway lumen.

Fig 2

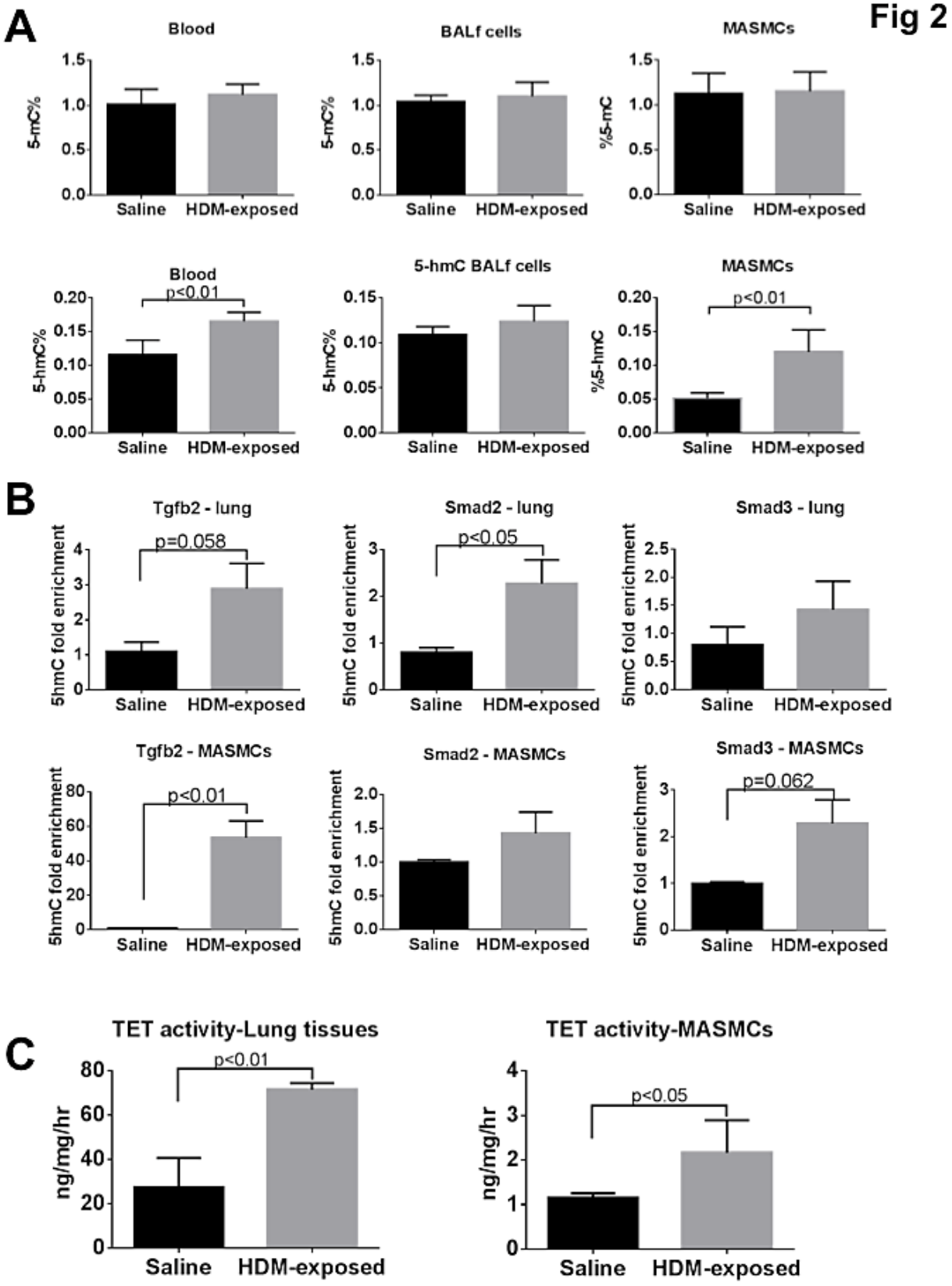
A
Blood
BALf cells
MASMCs
5-mC%
%5-mC
Saline
HDM-exposed
Blood
p<0.01
5-hmC BALf cells
MASMCs
5-hmC%
%5-hmC
B
Tgfb2 - lung
p=0.058
Smad2 - lung
p<0.05
Smad3 - lung
5hmC fold enrichment
Tgfb2 - MASMCs
p<0.01
Smad2 - MASMCs
Smad3 - MASMCs
p=0.062
C
TET activity-Lung tissues
p<0.01
TET activity-MASMCs
p<0.05
ng/mg/hr

Figure 2. Mice exposed chronically to HDM showed increased global and gene-specific DNA hydroxymethylation, and increased Tet activity in the lung tissues. (A) Levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in the DNA samples from the blood, BALF cells, and isolated tracheal ASM cells (MASMCs) were detected via ELISA. The results are expressed as the mean values ± SEM, with 4-5 mice per group. **(B)** Enrichment of 5hmC on *Tgfβ2, Smad2, and Smad3* were measured by qPCR. Hydroxymethylated DNA was immunoprecipitated with an antibody specific for 5hmC. After reverse-crosslinking, the DNA was subjected to qPCR to examine the enrichment of 5hmC at the promoter region of the gene of interest. β-actin was applied as a housekeeping gene to normalize the amount of input DNA. The fold enrichment was determined as the ratio to the level of enrichment of 5hmC in the saline-exposed group. The results are expressed as the mean values ± SEM, with 3-4 mice per group. **(C)** TET activity was measured via ELISA, which measures the rate of conversion of 5mC to 5hmC by Tet proteins. The results are the mean values ± SEM, with 4 or 3 mice per lung and mASMC group, respectively. For all comparisons, the unpaired t test was used, and statically significant comparisons were noted.

Tet1 Deficient Mice were protected from Acute HDM-induced AHR

We sought to determine whether Tet1 up-regulation is associated with HDM-induced AHR. First, 6-8 week-old male wild-type (WT) and heterozygous Tet1 deficient (*Tet1*$^{+/-}$) mice were subjected to HDM challenges for the assessment of AHR phenotypes. The HDM exposure significantly increased the airway reactivity (~140%) to the MCh challenge at 30 μg/mL of MCh (Figure 3A) in the WT mice. In comparison, the TET1 deficient mice exposed to HDM showed a significant decrease in airway reactivity to the MCh challenge. Next, we examined whether a Tet1 deficiency protected the tissues from the HDM-induced IgE production. Comparing the WT and *Tet1*$^{+/-}$ mice, the serum levels of IgE were unaffected by the loss of Tet1 in the acute HDM treated groups (Figure 3B). As expected, HDM exposure induced an inflammatory immune response by increasing eosinophils and lymphocytes, and decreasing the monocytes in the BALf in the WT mice (Figure 3C). In contrast, the TET1 deficiency did not result in a reduction in the HDM-induced eosinophilia. However, the HDM-exposed *Tet1*$^{+/-}$ mice showed a slight reduction in the number of lymphocytes (not statistically significant). In addition, we examined the mRNA levels of the inflammatory mediators in the lung. The levels of *Il4, Il13*, and *F*oxp*3* (but not *Il5, Il10*, and *Muc5b*) increased in both the WT and *Tet1*$^{+/-}$ mice exposed to HDM (Figure 2D). The Tet1 deficiency did not reduce the HDM-induction of these mediators.

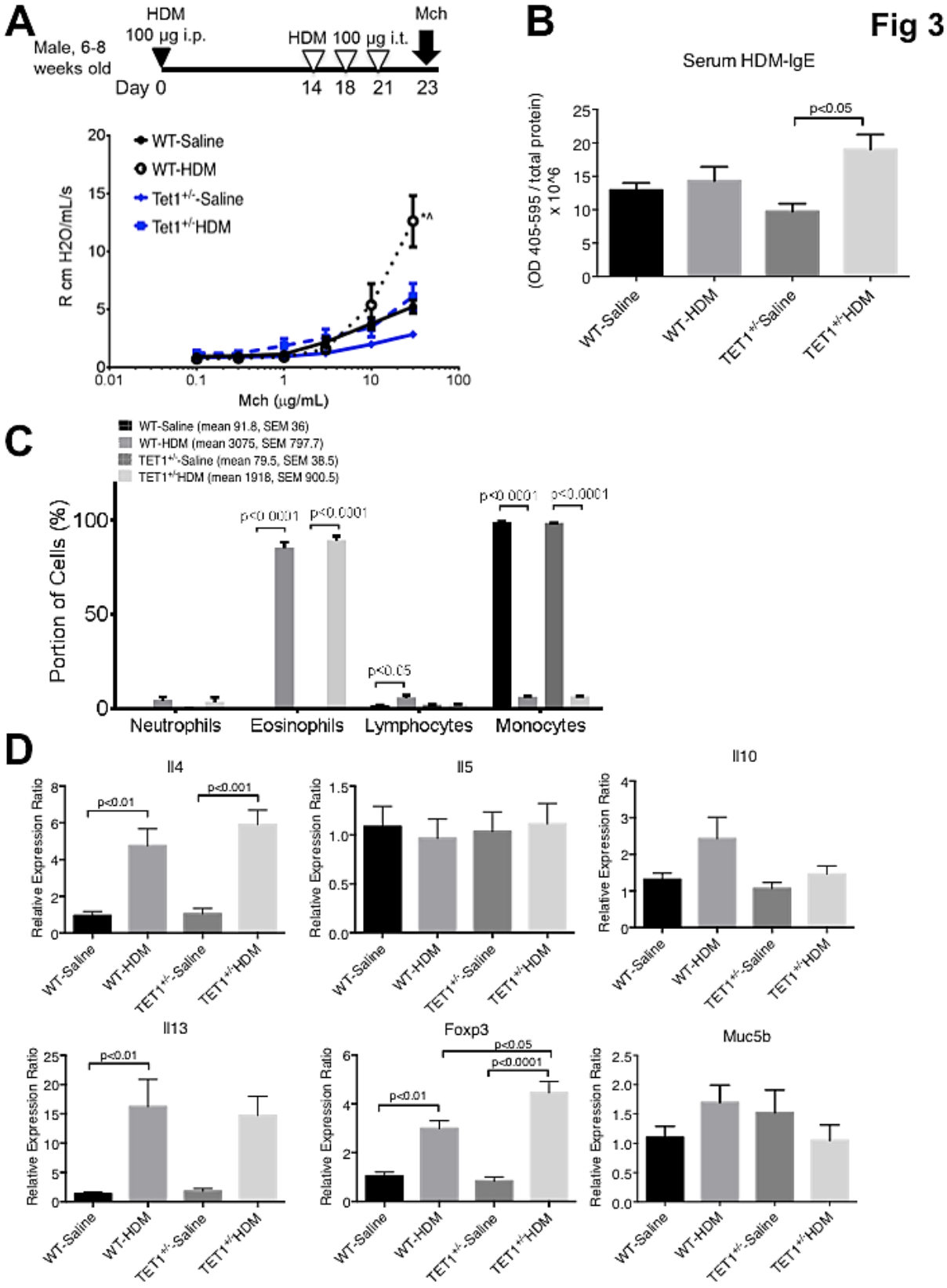
A
Male, 6-8 weeks old
HDM 100 µg i.p.
HDM 100 µg i.t.
Mch
Day 0
14 18 21 23
WT-Saline
WT-HDM
Tet1+/--Saline
Tet1+/-HDM
R cm H2O/mL/s
Mch (µg/mL)
B
Serum HDM-IgE
(OD 405-595 / total protein) x 10^6
p<0.05
WT-Saline
WT-HDM
TET1+/-Saline
TET1+/-HDM
C
WT-Saline (mean 91.8, SEM 36)
WT-HDM (mean 3075, SEM 797.7)
TET1+/--Saline (mean 79.5, SEM 38.5)
TET1+/-HDM (mean 1918, SEM 900.5)
Portion of Cells (%)
p<0.0001
p<0.0001
p<0.05
p<0.0001
p<0.0001
Neutrophils
Eosinophils
Lymphocytes
Monocytes
D
Il4
Il5
Il10
Il13
Foxp3
Muc5b
Relative Expression Ratio
p<0.01
p<0.001
p<0.05
p<0.0001
WT-Saline
WT-HDM
TET1+/--Saline
TET1+/-HDM

Fig 3

Figure 3. Tet1 deficiency reduced the acute HDM-induced airway hyper-responsiveness (AHR), which might not affect the allergen sensitization to HDM and the development of a Th2 immune response. (A) Upper panel: Schematic picture depicting the HDM sensitization and challenges used in this study. **Lower panel:** Methacholine (MCh) was administered to the animals at increasing doses (0.1, 0.3, 1, 3, 10, and 30 mg/mL) by a 10 second aerosol inhalation. AHR was assessed as the change in pulmonary resistance (R, $cmH_20/mL/s$) compared to the baseline after the challenge to MCh. The results are expressed as the mean values ± SEM with 9-12 mice per group. black box: wild-type (WT) saline-exposed (n=12), black circle: WT HDM-exposed (n=11), blue diamond: *Tet1*$^{+/-}$ saline-exposed (n=10), blue box: *Tet1*$^{+/-}$ HDM-exposed (n=9). The two-way ANOVA with Bonferroni's multiple comparisons test was used to determine the p values, which were *$p<0.05$ against the WT saline-exposed group, and ^ $p<0.05$ against the *Tet1*$^{+/-}$ HDM-exposed group. **(B)** The expression level of the HDM-specific IgE in the serum was measured via ELISA, and normalized to the total protein level. The results are expressed as the mean values ± SEM, with 6-9 mice per group. The one-way ANOVA with Tukey's multiple comparisons tests was used, and statically significant comparisons were noted. **(C)** The total cell counts were measured in the BALF. The results were the mean values (x10^4) ± SEM, with 6-10 mice from each group, and presented next to the legends. WT saline-exposed (n=8), WT HDM-exposed (n=10), *Tet1*$^{+/-}$ saline-exposed (n=6), *Tet1*$^{+/-}$ HDM-exposed (n=6). Differential cell counts were obtained under a light microscope after staining the BAL cells with Hema-3 stain. The portion of the immune cells was calculated by the ratio in 400 cells counted per slide, multiplied by the total number of cells. The results were the mean values ± SEM, with 6-7 mice from each group. WT saline-exposed (n=6), WT HDM-exposed (n=6), *Tet1*$^{+/-}$ saline-exposed (n=7), *Tet1*$^{+/-}$ HDM-exposed (n=6). The two-way ANOVA with Fisher's LSD tests was used, and statically significant comparisons were noted. **(D)** The mRNA levels of the inflammatory mediators were assayed by qPCR. The results were the mean values ± SEM, with 5-6 mice from each group. The one-way ANOVA with Bonferroni's multiple comparisons tests was used, and statically significant comparisons were noted.

Next, we investigated the effects of the Tet1 deficiency upon the pathological changes in the lungs from HDM exposure. First, H&E staining confirmed the findings from the differential cell count (Figure 3C), of increased immune cell infiltration into the lungs of the WT mice exposed to HDM, compared to those of the saline-exposed mice (Figure 4A). A slightly decreased cell infiltrate was shown in the HDM-exposed *Tet1*$^{+/-}$ mice. Second, Mason's trichrome staining (MTS) revealed significant increases in the collagen deposition, especially around the airways, in the lungs of HDM-exposed WT mice. However, we did not observe a significant reduction in the collagen deposition in the lungs of the *Tet1*$^{+/-}$ mice exposed to HDM (Figure 4B). Finally, the HDM-exposed WT mice showed an increased number of goblet cells in the bronchial epithelium. The Tet1 deficient mice had slightly reduced the numbers of goblet cells and mucus formation (although not shown in the saline control groups), as shown by the Periodic Acid Schiff staining (Figure 4C). The histological assessment of the lung sections indicates that the loss of Tet1 activity caused a slight decrease in the HDM-induced airway inflammation. Overall, the loss of the Tet1 activity protected the mice from HDM-induced AHR, but it did not appear to affect the development of a Th2 immune response and allergen sensitization to HDM. Therefore, we proposed determining whether Tet1 regulates allergic AHR by altering the expression of the genes directly affecting ASM function.

To further investigate the role of Tet1 in acute HDM-induced AHR phenotypes, we measured changes in the ASM phenotypic genes in both the lungs and MASMCs. The HDM-exposed WT mice showed increases in the mRNA levels of *Ccnd1*, *Pcna* (cell proliferation) *Camk2D*, and *Mlck* (cell contraction), but not *Col1a* and *Col3a* (collagen synthesis) in their lung tissues, compared to those of the WT saline control (Figure 5A).

Therefore, the Tet1 deficiency could completely prevent the HDM-induction of Pcna, and attenuate the two-fold induction of *Mlck*. When evaluating the ASM phenotypic genes in the MASMCs, the HDM-exposed WT mice showed a trend toward increased expressions of *Pcna*, *Col3a* and *Col1a* (although not statistically significant, p=0.08) (Figure 5B). The loss of Tet1 in HDM-exposed *Tet1*$^{+/-}$ mice completely prevented the HDM-induction of *Pcna* and *Col1a* but not *Col3a*. These date demonstrate that Tet1 deficiency provided protection from HDM-induced AHR by modulating the expression of genes related to the proliferation, contraction, and collagen phenotypes of the ASMCs.

Fig 4

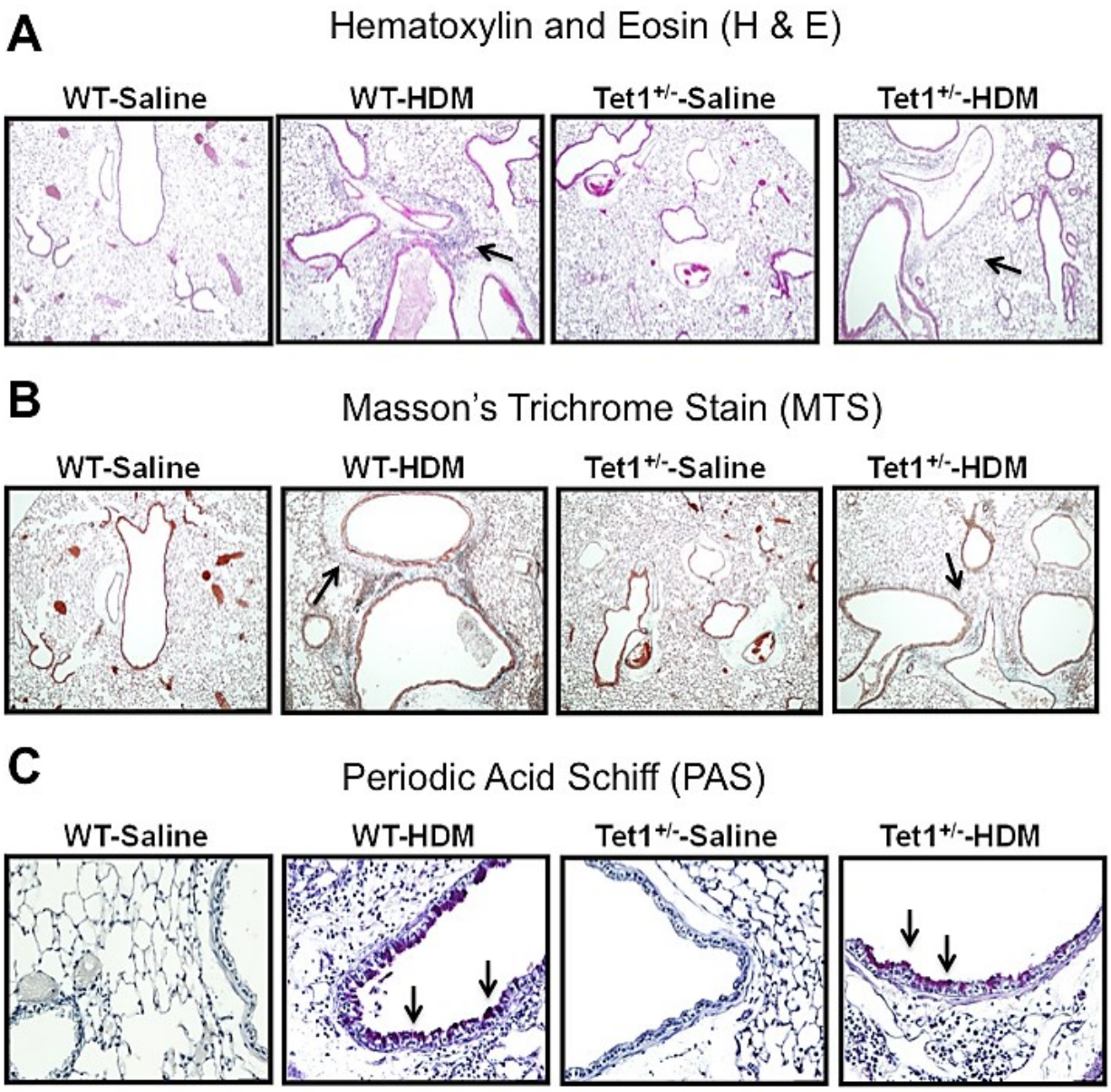

Figure 4. *Tet1*$^{+/-}$ deficient mice showed a slight decrease in the HDM-induced airway inflammation. The lung sections were stained with **(A)** Hematoxylin and Eosin (H&E) (scale bar = 100 μm), **(B)** Masson's Trichrome Stain (MTS) (scale bar = 100 μm), and **(C)** Periodic acid-Schiff (PAS) (scale bar = 50 μm). The representative images of the lungs from each group are presented. The arrows indicate **(A)** Increased inflammatory infiltration was observed near the airways of the WT HDM-exposed mice, with a slight decrease in the *Tet1*$^{+/-}$ HDM-exposed mice. **(B)** A slight decrease in collagen production (collagen fiber stained in blue) and **(C)** formation of goblet cells in the epithelium of the bronchus (stained in purple) in *Tet1*$^{+/-}$ HDM-exposed mice, compared to the WT HDM-exposed mice.

Fig 5

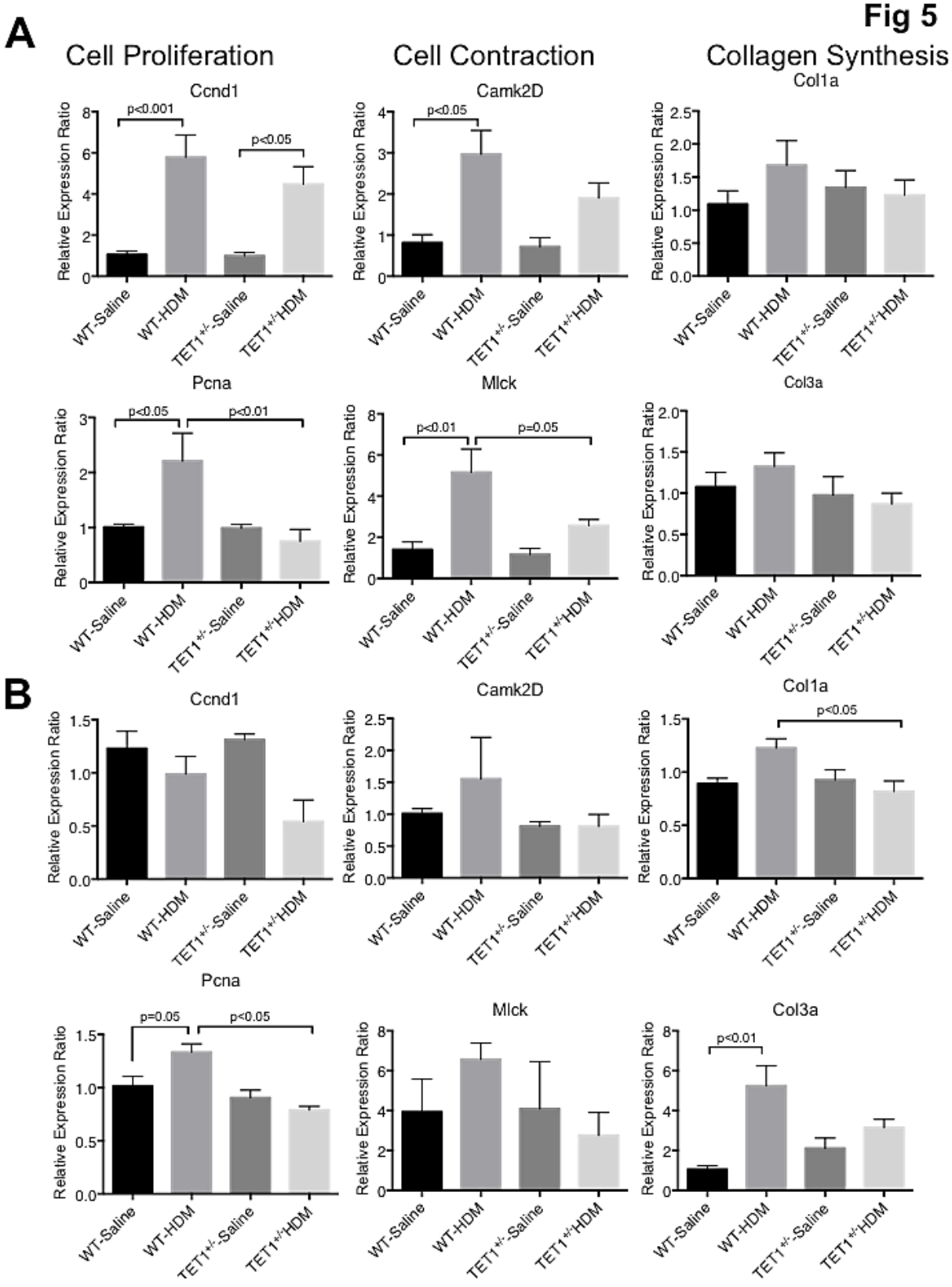
A
Cell Proliferation
Cell Contraction
Collagen Synthesis
Ccnd1
Camk2D
Col1a
Pcna
Mlck
Col3a
Relative Expression Ratio
p<0.001
p<0.05
p<0.01
p=0.05
WT-Saline
WT-HDM
TET1+/--Saline
TET1+/-HDM
B
Ccnd1
Camk2D
Col1a
Pcna
Mlck
Col3a
p=0.05
p<0.05
p<0.01

Figure 5. Tet1 suppression prevented the HDM-induction of *Pcna* and *Mlck* in the lung tissues, and *Pcna* and *Ccnd1* in the isolated ASMCs. The mRNA level of the ASM phenotypic genes (involved in cell proliferation and collagen synthesis) in the **(A)** lung tissues and **(B)** isolated ASMCs were measured by qPCR. The results were the mean values ± SEM (n=6 for lung tissues and n=3-6 for ASMCs per group). The one-way ANOVA with Bonferroni's multiple comparisons tests were used, and statistically significant comparisons were noted.

Loss of Tet1 modifies DNA Methylation Changes induced by HDM Exposure

We further tested our hypothesis that Tet1 up-regulation contributes to allergic AHR through DNA methylation changes in the lung. We found no significant changes in the lung 5mC content among any of the treatment groups (Figure 6A). But in the mouse ASMCs HDM exposure decreased the global 5mC content in both the WT and *Tet1*$^{+/-}$ mice (Figure 6A). Although, there was no change in 5mC content in the mouse ASMCs between the WT and Tet1 deficient mice exposed to HDM. In comparison, 5hmC content in mouse ASMCs decreased in Tet1 deficient mice exposed to HDM, compared to the WT-HDM group (Figure 6B). The results indicated that the Tet1 could modulate global changes in the DNA hydroxymethylation induced by HDM exposure in both the lung tissues and mouse ASMCs.

Fig 6

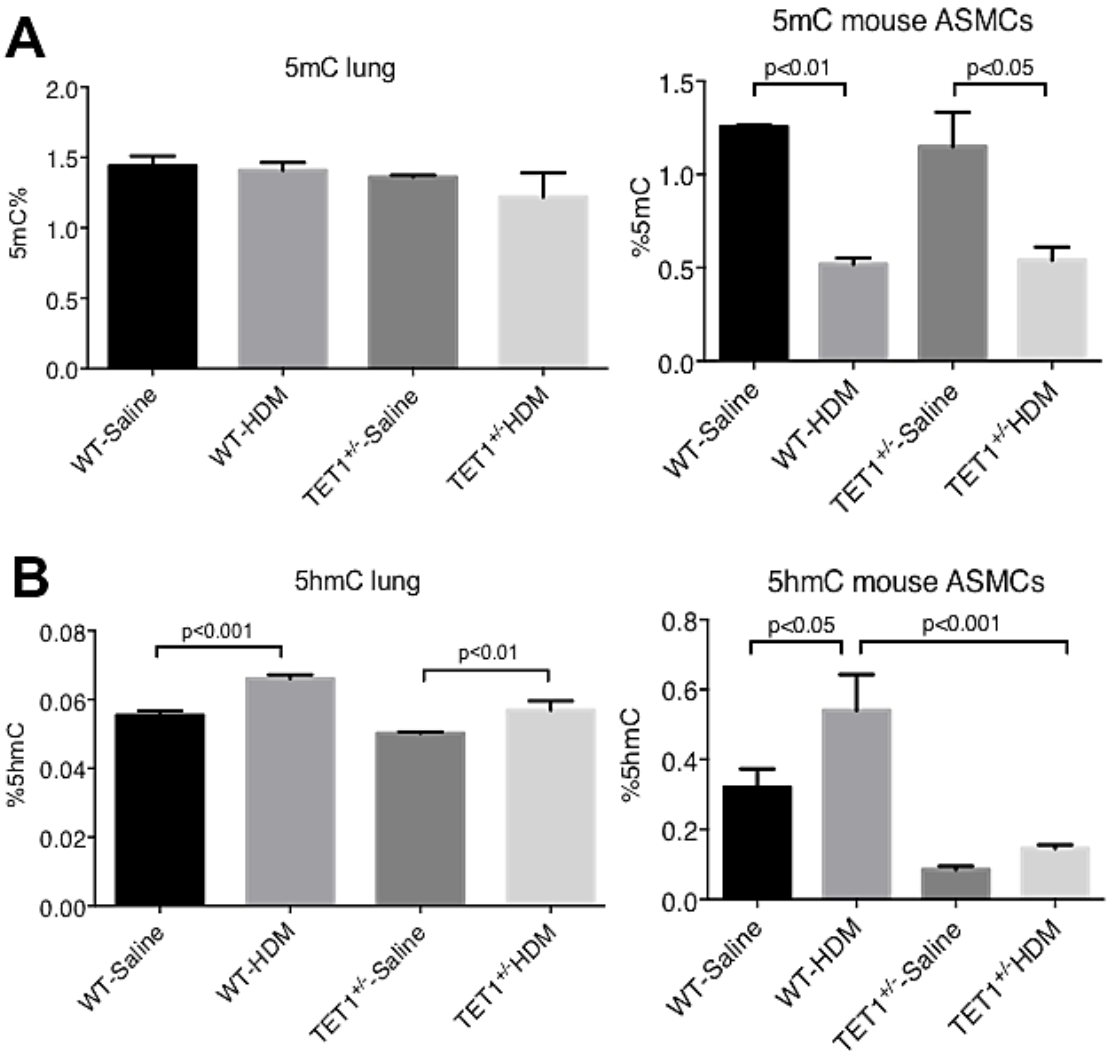

Figure 6. The Tet1 deficiency decreased the HDM-induced global DNA hydroxymethylation in the isolated ASM cells. The levels of **(A)** 5mC and **(B)** 5hmC in DNA samples from the lung tissues and isolated tracheal ASMCs were detected via ELISA. The results were the mean values ± SEM (n=3-6 for lung tissues and n=3 for ASMCs per group). The one-way ANOVA with Bonferroni's multiple comparisons test was used, and significant changes were noted.

In addition to the global changes in the DNA methylation and hydroxymethylation, we examined whether Tet1 up-regulation contributed to the HDM-induced gene-specific DNA methylation changes in the mouse lungs and ASMCs. By utilizing genome-wide DNA methylation profiling techniques, our group identified differentially methylated candidates in the HDM-exposed mouse lung and ASMCs, and these changes were associated with an increased HDM-induced AHR (45). First, we examined the gene expression changes of these differentially methylated candidates in the mouse lung tissues (Table 2). The HDM-exposed WT mice showed a significant increase in mRNA level of *Cartpt, Pde4d, Pom121l2, Smad3*, and *Tgfβ2*. A non-significant increase in *Slc25a12* and *Trpm2* (p=0.07) in these mice was also observed. The loss of Tet1 activity could completely reverse the HDM-induction of *Pde4d, Slc25a12 and Trpm2*. A 50% decrease in the *Tgfβ2* expression was also found in the HDM-exposed $Tet1^{+/-}$ mice. We further examined whether the reduction of the *Pde4d, Slc25a12, Tgfβ2* and *Trpm2* were concomitant with the changes in their methylation status (Table 3). We found that the Tet1 deficiency reduced the HDM-induction of the *Pde4d* concomitant with increased *Pde4d* promoter methylation. In addition, the loss of Tet1 activity increased the *Akt1s1* promoter methylation when compared to the WT mice, although it did not result in silencing the *Akt1s1*.

Table 2: Changes in mouse lung mRNA levels of genes that previously showed differential methylation in HDM-induced AHR models (*Shang Y et al. AJRCMB 2013, Cheng RYS et al. EMM 2014*).

Gene Symbol	Gene Name	WT Saline-exposed		WT HDM-exposed		Tet1$^{+/-}$ Saline-exposed		Tet1$^{+/-}$ HDM-exposed	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Akt1s1	AKT1 substrate 1	1.10	0.20	1.50	0.26	1.40	0.27	0.80	0.06
Bmp3	Bone morphogenetic protein 3	1.07	0.17	1.73	0.16	1.35	0.17	1.15	0.13
Cartpt	CART prepropeptide	1.13	0.23	4.32***	0.58	0.65###	0.14	4.24	0.37
Il6r	Interleukin 6 Receptor	1.12	0.22	1.32	0.32	1.08	0.14	1.13	0.19
Pde4d	Phosphodiesterase 4D, cAMP specific	1.08	0.16	2.36***	0.25	0.40	0.09	0.82^^^	0.12
Pom121l2	POM121 Transmembrane Nucleoporin-Like 2	1.20	0.32	4.64***	0.36	0.77###	0.21	4.84	0.32
Slc25a12	Solute carrier family 25, member 12	1.06	0.19	1.17	0.06	0.59	0.14	0.70^	0.07
Smad3	MAD homolog 3 (Drosophila)	0.87	0.10	1.61***	0.16	1.08##	0.07	1.82	0.09
Tgfb2	Transforming Growth Factor, Beta 2	1.00	0.05	1.92***	0.10	1.10	0.13	1.36^^	0.07
Trpm2	Transient Receptor Potential Cation Channel, Subfamily M, Member 2	1.03	0.11	1.28	0.22	1.15	0.31	0.36^	0.06

Note:
mRNA level of methylated candidates in lung tissues were measured by qPCR.
Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where RER s set as 1.00 in the WT Saline-exposed group.
Results expressed as mean values ± SEM (n=5-6 mice per group).
One-way ANOVA with Bonferroni's multiple comparisons tests used.
*p<0.05, **p<0.01 or ***p<0.001 vs WT Saline-exposed
#p<0.05, ##p<0.01 or ###p<0.001 vs Tet1$^{+/-}$ HDM-exposed
^ p<0.05, ^^p<0.01 or ^^^p<0.001 vs WT HDM-exposed

Table 3: Promoter methylation changes in mouse lung genes that previously showed differential methylation in HDM-induced AHR models (*Shang Y et al. AJRCMB 2013, Cheng RYS et al. EMM 2014*).

Gene Symbol	WT Saline-exposed		WT HDM-exposed		Tet1$^{+/-}$ Saline-exposed		Tet1$^{+/-}$ HDM-exposed	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Akt1s1	20.92	4.09	28.05$_{(n=4)}$	7.86	28.37$^{###}$	6.08	69.30$^{\wedge\wedge\wedge}{}_{(n=3)}$	6.31
Bmp3	6.43	1.90	7.51	0.80	8.94	3.07	3.49	0.66
Pde4d	8.05	2.19	6.20	1.79	7.88$^{\#}$	1.72	14.80$^{\wedge}$	3.03
Slc25a12	3.33	2.18	1.17$_{(n=4)}$	0.38	1.75$_{(n=2)}$	0.13	0.95$_{(n=3)}$	0.45
Tgfb2	13.71$_{(n=4)}$	2.64	14.63$_{(n=4)}$	1.60	9.56	3.15	11.08	3.10
Trpm2	5.17	1.27	6.24	1.63	7.08	1.03	6.69	1.21

Note:

Promoter percent methylation of candidate genes in the lung tissues were measured by MSPCR.

Levels of methylated DNA were densitometrically analyzed (GelQuantNET) and standardized to β-actin.

Results are expressed as mean values ± SEM (n=5-6 mice per group; unless noted otherwise).

One-way ANOVA with Fisher's test was used and significant changes were noted.

*p<0.05, **p<0.01 or ***p<0.001 vs WT Saline-exposed

$^{\#}$p<0.05, $^{\#\#}$p<0.01 or $^{\#\#\#}$p<0.001 vs Tet1$^{+/-}$ HDM-exposed

^ p<0.05, ^^p<0.01 or ^^^p<0.001 vs WT HDM-exposed

Next, we examined whether the loss of the Tet1 activity modulated the gene-specific expression and methylation changes in ASMCs. In comparison, the ASMCs from the HDM-exposed WT mice showed increased transcript levels of *Bmp3, Cartpt Pde4d, Smad3*, *Tgfβ2* and *Trpm2* (Table 4). Interestingly, the Tet1 deficiency in the ASMCs almost completely blocked the HDM induction of the *Bmp3, Cartpt, Smad3*, *Tgfβ2* and *Trpm2*. A 70% reduction in the HDM-induced Pde4d up-regulation was found in the HDM-exposed *Tet1*$^{+/-}$ mice. In addition, a decrease in the gene expression levels was shown in accordance with the increased DNA methylation status (Table 5). These results indicate that the loss of Tet1 activity can prevent the HDM induction of gene expression and gene demethylation, specifically in ASMCs.

Table 4: Changes in mouse ASM cells mRNA levels of genes that previously showed differential methylation in HDM-induced AHR models (*Shang Y et al. AJRCMB 2013, Cheng RYS et al. EMM 2014*).

Gene Symbol	WT Saline-exposed		WT HDM-exposed		Tet1$^{+/-}$ Saline-exposed		Tet1$^{+/-}$ HDM-exposed	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Akt1s1	1.07	0.24	1.30	0.23	1.38	0.16	1.43	0.49
Bmp3	1.14	0.05	1.67***	0.13	0.98	0.02	1.20^^	0.05
Cartpt	1.15	0.46	2.08***	0.20	1.15	0.11	1.27^^	0.11
Il6r	0.91	0.05	1.16	0.10	1.04	0.09	0.88	0.10
Pde4d	1.17	0.37	7.06**	1.10	2.92	1.10	2.13^	0.59
Pom121l2	1.66	1.30	2.65	0.76	3.36	1.10	4.40	0.35
Slc25a12	1.17	0.05	1.12	0.06	0.82	0.06	0.90	0.11
Smad3	1.00	0.13	1.91**	0.22	1.20	0.13	1.13^^	0.05
Tgfb2	1.03	0.04	2.55***	0.25	1.21	0.12	1.05^^^	0.08
Trpm2	0.99	0.07	4.15***	0.72	1.14	0.12	1.42^^^	0.18

Note:

mRNA level of methylated candidates in the tracheal isolated mouse airway smooth muscle (ASM) cells were measured by qPCR.

Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the WT Saline-exposed group.

Results expressed as mean values ± SEM (n=6 mice per group).

One-way ANOVA with Bonferroni's multiple comparisons tests used.

*p<0.05, **p<0.01 or ***p<0.001 vs WT Saline-exposed

$^{\#}$p<0.05, $^{\#\#}$p<0.01 or $^{\#\#\#}$p<0.001 vs Tet1$^{+/-}$ HDM-exposed

^ p<0.05, ^^p<0.01 or ^^^p<0.001 vs WT HDM-exposed

Table 5: Promoter methylation changes in mouse ASM cells genes that previously showed differential methylation in HDM-induced AHR models (*Shang Y et al. AJRCMB 2013, Cheng RYS et al. EMM 2014*).

Gene Symbol	WT Saline-exposed		WT HDM-exposed		Tet1$^{+/-}$ Saline-exposed		Tet1$^{+/-}$ HDM-exposed	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Bmp3	33.01	3.87	4.81*	1.50	10.47	4.32	31.78^	13.49
Cartpt	72.57	7.31	62.34	4.52	76.03	2.35	92.87^^	6.61
Pde4d	61.58	8.24	31.26**	2.95	47.59	3.45	60.91^^	8.01
Smad3	65.63	3.80	39.98*	3.47	60.22	5.01	70.90^^	8.62
Tgfb2	30.54	5.15	10.06*	2.69	21.74	9.65	22.32	2.01
Trpm2	39.68	2.31	11.16**	0.65	54.95$^{###}$	6.12	17.34	5.36

Note:

Promoter percent methylation of candidate genes in the tracheal isolated mouse airway smooth muscle (ASM) cells were measured by MSPCR.

Levels of methylated DNA were densitometrically analyzed (GelQuantNET) and standardized to β-actin.

Results are expressed as mean values ± SEM (n=3 mice per group; unless noted otherwise).

One-way ANOVA with Fisher's test was used and significant changes were noted.

*p<0.05, **p<0.01 or ***p<0.001 vs WT Saline-exposed

$^{\#}$ p<0.05, $^{\#\#}$p<0.01 or $^{\#\#\#}$p<0.001 vs Tet1$^{+/-}$ HDM-exposed

^ p<0.05, ^^p<0.01 or ^^^p<0.001 vs WT HDM-exposed

Inhibition of TET1 modulates the Aberrant Cell Phenotypes in Asthmatics

To assess potential clinical relevance, we investigated the epigenetic role of TET1 in regulating the cell functions in human ASMCs. To determine whether TET1 dysregulation resulted in altered ASM cell proliferative and contractile responses in asthmatics, we compared these ASM indices in ASMCs isolated from the deceased lung donations of non-asthmatics and asthmatics (Table 6). Asthmatic ASMCs had increased gene expressions of *CCND1* and *PCNA* (cell proliferation), *CAMK2D* (cell contraction), *C*ol*1A* (collagen synthesis) and *ADAM33, IL1B, SMAD2, SMAD3*, *TGFβ2* and *VEGF* (growth factors, cytokines, and mediators) compared to ASMCs from non-asthmatics (Table 7). These data suggest that asthmatic ASMCs can generate more collagens, growth factors, cytokines, and mediators that make the cells more proliferative and contractile than non-asthmatic ASMCs. Notably, the human asthmatic ASMCs showed several features that were similar to the tracheal ASMCs isolated from the AHR mice.

Table 6. Characteristics of non-asthmatic and asthmatic ASM cell donors.

Label ID	AGE	GENDER	RACE	HISTORY
Non-asthmatic Airway Smooth Muscle (ASM) Cells				
NOL	27	Male	Not disclosed	Not disclosed (LONZA, MD)
NO1	18	Female	Caucasian	Head trauma, smoked 1/3 pack per day for 4 months
NO2	17	Male	Caucasian	Gunshot wound to head, smoked unknown amount 3 days a week
NO3	24	Female	Caucasian	Cardiac arrest, no tobacco use
NO4	31	Female	Black	Aneurysm, tobacco use for 10 years
NO7	22	Male	Black	Head trauma, 1 cigarette daily for 1 year
NO13	17	Male	Black	Gunshot wound to head, no tobacco use
NO14	48	Male	Hispanic	Stroke, no tobacco use
Label ID	**AGE**	**GENDER**	**RACE**	**HISTORY**
Asthmatic Airway Smooth Muscle (ASM) Cells				
ASL	4	Male	Not disclosed	Not disclosed (LONZA, MD)
AS1	18	Female	Caucasian	Anoxia, asthma attack, no tobacco use
AS2	14	Male	Caucasian	Anoxia, asthma attack, no tobacco use
AS3	27	Female	Caucasian	Anoxia, asthma attack, smoked 5 times daily for 7 years; Quit 2 months ago
AS6	38	Male	Caucasian	Anoxia, asthma attack, no tobacco use
AS7	35	Female	Caucasian	Anoxia, no tobacco or drug use

Note:
Except NOL and ASL, all other human ASM cells were derived from tracheas of deceased asthmatic and non-asthmatic donors from the National Disease Research Interchange (Philadelphia, PA), as previously described (*Robinett et al. 2014*).

Table 7. mRNA levels of ASM phenotypic genes in human non-asthmatic and asthmatic ASM cells.

Gene Symbol	Gene Name	Non-asthmatics		Asthmatics		p-value
		Mean	SEM	Mean	SEM	
Cell Proliferation/Cell Mass						
CCND1	Cyclin D1	1.35	0.34	4.70	1.28	0.03*
PCNA	Proliferating Cell Nuclear Antigen	1.10	0.06	1.59	0.15	0.01*
SMA	Smooth Muscle Actin	1.33	0.17	2.30	0.60	0.16
Cell Contraction						
CAMK2D	Calcium/Calmodulin-Dependent Protein Kinase II Delta	1.30	0.12	2.64	0.60	0.03*
MYLK2	Myosin Light Chain Kinase 2	0.92	0.13	1.65	0.34	0.06
Collagen Synthesis						
COL1A	Collagen, Type I, Alpha 1	1.28	0.21	2.75	0.61	0.05*
COL3A	Collagen, Type III, Alpha 1	1.83	0.50	2.54	0.44	0.31
Growth Factors, Cytokines and Mediators						
ADAM33	ADAM Metallopeptidase Domain 33	1.16	0.29	4.35	1.35	0.05*
CSF2	Colony Stimulating Factor 2	1.00	0.05	0.77	0.05	0.79
ICAM1	Intercellular Adhesion Molecule 1	1.08	0.18	1.88	0.59	0.16
IL1B	Interleukin 1, Beta	1.08	0.18	3.11	0.56	<0.01**
IL4	Interleukin 4	1.02	0.09	0.82	0.04	0.15
IL6	Interleukin 6	1.05	0.17	1.37	0.11	0.16
IL8	Interleukin 8	1.18	0.34	1.16	0.18	0.97
SMAD2	SMAD Family Member 2	1.55	0.21	3.01	0.64	0.05*
SMAD3	SMAD Family Member 3	1.28	0.11	2.23	0.37	0.03*
TGFB2	Transforming Growth Factor, Beta 2	0.85	0.08	2.25	0.52	0.02*
TNFA	Tumor Necrosis Factor-Alpha	1.15	0.32	0.46	0.13	0.06
VCAM1	Vascular Cell Adhesion Molecule 1	1.05	0.14	1.07	0.12	0.54
VEGF	Vascular Endothelial Growth Factor	1.16	0.30	4.87	1.97	0.03*

Note:

mRNA levels of genes of interests in human airway smooth muscle (ASM) cells were measured by qPCR.

Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method where the RER set as 1.00 in the non-asthmatic group.

Results are expressed as mean values ± SEM, with 5 donors per group.

Unpaired t-tests were used to determine the p-value comparing non-asthmatics and asthmatics ASM cells.

*p<0.05 or **p<0.01

Furthermore, we showed that the ASMCs from the asthmatic donors had a 50% increase in the 5mC content and 80% increase in the 5hmC content (Figures 7A and 7B). In addition, the asthmatic ASMCs had twice the level of TET1 mRNA and TET activities when compared to those of the non-asthmatic ASMCs (Figures 7C and 7D).

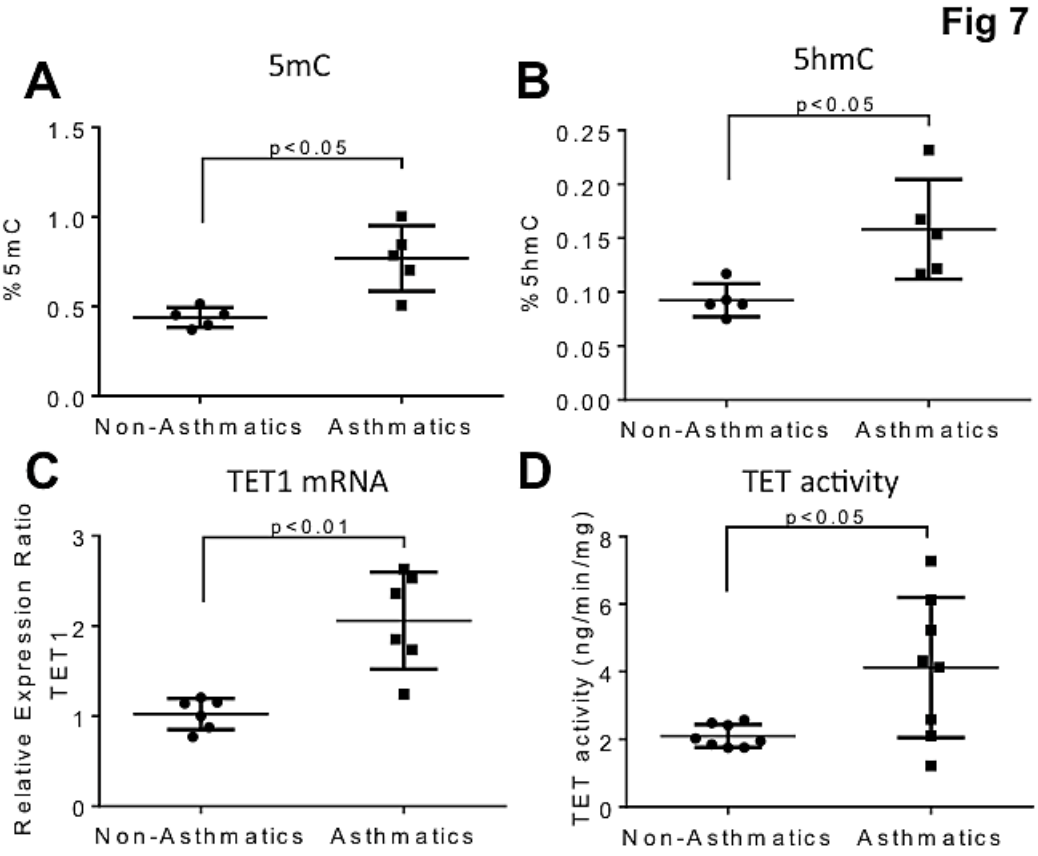

Figure 7. Asthmatic ASMCs showed increased 5mC, 5hmC, *TET1* mRNA level, and TET activity compared to the non-asthmatic ASMCs. The levels of **(A)** 5mC and **(B)** 5hmC in the DNA samples isolated from the non-asthmatic and asthmatic ASMCs were detected using ELISA. **(C)** The mRNA levels of *TET1* and **(D)** TET activity were measured by qPCR and ELISA, respectively. The results were the mean values ± SEM. With (A) n=5, (B) n=5, (C) n=6, and (D) n=8 donors from non-asthmatics and asthmatics. The unpaired t-test with Welch's correction was performed, and statistically significant comparisons were noted.

To directly determine whether the TET1-mediated epigenetic changes are associated with abnormal ASM cell function (increased ASM cell proliferation, contraction and collagen synthesis) in asthmatics, we utilized small interfering RNAs (siRNAs) against TET1 (si*TET1*) to target the knockdown of *TET1* in both non-asthmatic and asthmatic ASMCs. si*CTL* was used as the non-target siRNA control in the si*TET1* transfection assay. We demonstrated that both si*TET1* and si*TET2* decreased *TET1* and *TET2* mRNA levels by more than 50%, respectively (Figures 8A and B), suggesting an effective siRNA knockdown in the ASMCs. The si*TET1* and si*TET2* decreased TET activity by 70% and 15%, respectively (Figure 8C). This suggests that the reduction in the TET activity was mainly due to TET1 in asthmatic ASMCs. To determine if TET1 played a role in modulating cell proliferation, we measured the BrdU incorporation in the ASMCs upon si*TET1* knockdown. As shown in other reports, the asthmatic ASM cells were more proliferative than the non-asthmatic ASMCs (Figure 8D). The knockdown of *TET1* caused a decrease in the cell proliferation in asthmatic ASMCs when compared to the siCTL group. However, there was no significant effects seen in the non-asthmatic ASMCs. To further examine whether *TET1* over-expression contributes to the aberrant phenotypes seen in asthmatics, we compared the expression levels of the ASM phenotypic genes by qPCR (Table 8) in the cells treated with si*TETl*.

In the asthmatic ASMCs, si*TET1* decreased the *PCNA, SMA* (cell proliferation/mass), *CAMK2D* (cell contraction), *ADAM33* and TGFB2 (growth factors) gene expressions when compared to the si*CTL* treatment. There were no significant differences in the ASM phenotypic gene expression in the non-asthmatic ASMCs treated

with si*TET1*. The absence of the silencing effect of the si*TET1* on the ASM phenotypic genes in non-asthmatic ASMCs suggests that their respective ASM phenotypic genes may be at very low abundance. Taken together, these results indicate that TET1 depletion could reverse the elevated expression of the ASM phenotypic genes and cell proliferation in asthmatics, suggesting the significance of TET1 in regulating the ASM phenotypes.

Fig 8

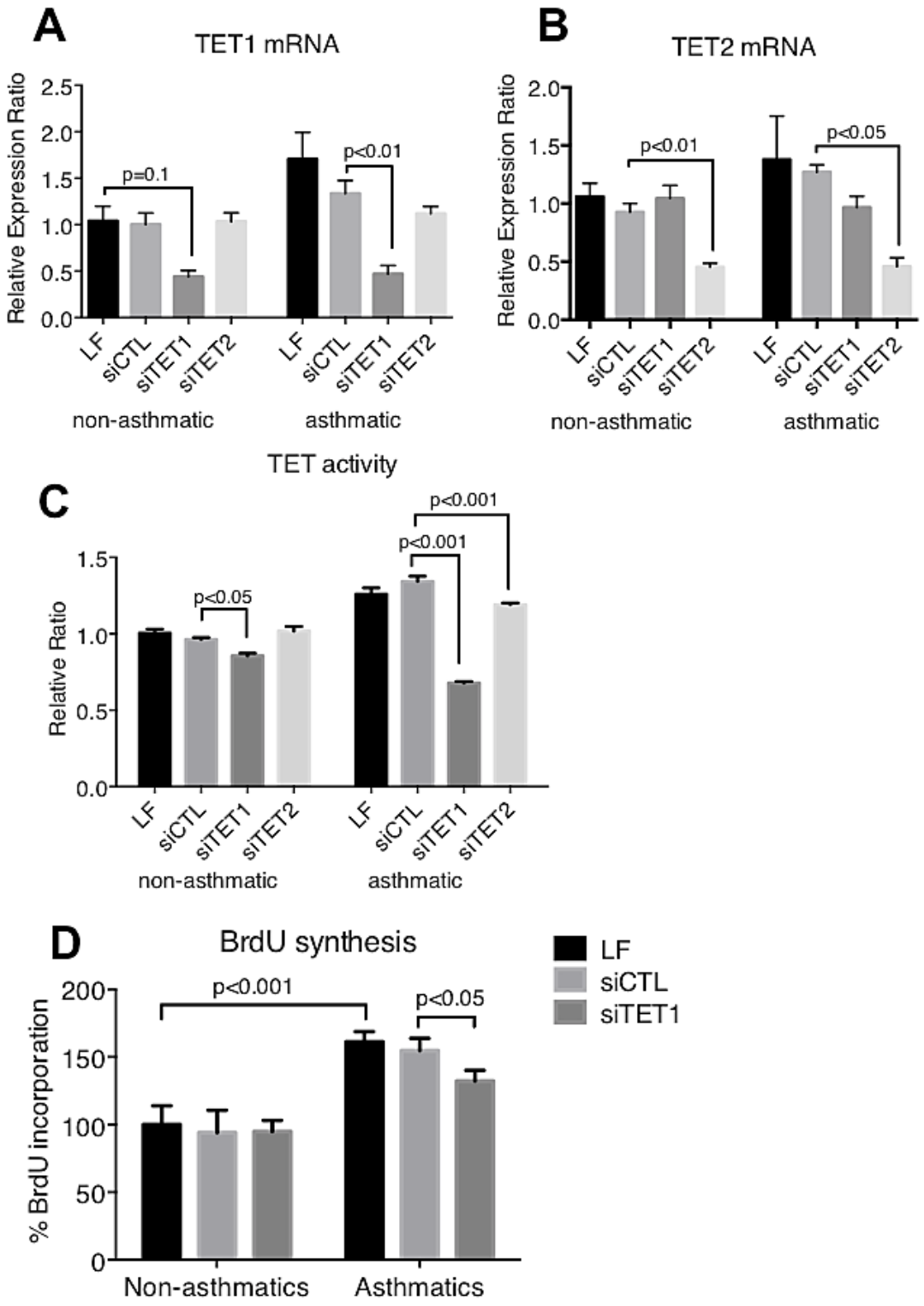
A
TET1 mRNA
Relative Expression Ratio
2.5
2.0
1.5
1.0
0.5
0.0
p=0.1
p<0.01
LF
siCTL
siTET1
siTET2
non-asthmatic
asthmatic
B
TET2 mRNA
Relative Expression Ratio
2.0
1.5
1.0
0.5
0.0
p<0.01
p<0.05
LF
siCTL
siTET1
siTET2
non-asthmatic
asthmatic
C
TET activity
Relative Ratio
1.5
1.0
0.5
0.0
p<0.05
p<0.001
p<0.001
LF
siCTL
siTET1
siTET2
non-asthmatic
asthmatic
D
BrdU synthesis
% BrdU incorporation
200
150
100
50
0
p<0.001
p<0.05
Non-asthmatics
Asthmatics
LF
siCTL
siTET1

Figure 8. si*TET1* knockdown decreases *TET1* expression and TET activity in ASMCs. The ASMCs were treated with lipofectamine (LF), non-targeting control si*CTL*, or si*TET1* or si*TET2* oligonucleotides for 3 days. The mRNA level of **(A)** *TET1* and **(B)** *TET2*, and the **(C)** TET activity were measured by qPCR and ELISA, respectively. The results were expressed as the mean values ± SEM, with 5 non-asthmatic and 5 asthmatic donors. A two-way ANOVA with Tukey's multiple comparisons tests was used, and statistically significant comparisons were noted. **(D)** The transfected cells were incubated with BrdU (5-bromo-2'-deoxyuridine) for 6 hours to allow the BrdU to be incorporated in place of thymidine into the newly synthesized DNA of the proliferating cells. The BrdU incorporation into the DNA was measured by colorimetric detection. The percent (%) BrdU incorporation was normalized to the LF control of the non-asthmatic ASMCs. The results were the mean values ± SEM, with 4 non-asthmatic donors and 4 asthmatic donors. The two-way ANOVA with Tukey's multiple comparisons and statically significant comparisons were noted.

Table 8. Changes in mRNA levels of ASM phenotypic genes in human ASM cells in response to siTET1 knockdown.

Gene Symbol	Non-Asthmatics							Asthmatics						
	LF[A]		siCTL		siTET1		pvalue (siTET1 vs siCTL)[B]	LF		siCTL		siTET1		pvalue (siTET1 vs siCTL)[C]
	Mean	SEM	Mean	SEM	Mean	SEM		Mean	SEM	Mean	SEM	Mean	SEM	
Cell Proliferation/Mass														
CCND1	0.83	0.13	1.06	0.07	1.24	0.09	> 0.99	1.59	0.33	1.72	0.32	1.06	0.14	0.18
PCNA	0.97	0.03	0.98	0.12	1.08	0.05	> 0.99	2.20	0.14	1.96	0.21	1.28	0.09	<0.01**
SMA	1.08	0.23	0.97	0.20	0.60	0.26	0.71	1.03	0.18	1.71	0.27	0.35	0.06	<0.01**
Cell Contraction														
CAMK2D	1.02	0.14	0.88	0.07	0.59	0.12	0.63	1.16	0.23	1.09	0.13	0.56	0.08	0.04*
MYLK2	1.02	0.05	1.01	0.26	0.67	0.10	0.39	1.42	0.12	1.13	0.16	0.87	0.03	0.41
Collagen Synthesis														
COL1A	1.08	0.19	1.21	0.12	0.94	0.31	> 0.99	1.28	0.19	1.18	0.17	0.81	0.07	0.31
COL3A	1.06	0.13	1.37	0.28	0.85	0.23	0.47	2.10	0.30	1.86	0.29	1.16	0.04	0.12
Growth Factors, Cytokines and Mediators														
ADAM33	1.07	0.39	1.71	0.98	0.64	0.27	0.51	1.16	0.43	2.38	0.39	0.55	0.21	0.04*
ICAM1	1.08	0.28	1.21	0.04	4.04	1.30	0.77	7.34	1.85	6.94	1.60	8.57	5.05	> 0.99
IL1B	1.09	0.32	1.75	0.70	3.19	0.32	0.10	1.40	0.51	0.86	0.29	1.38	0.20	> 0.99
IL6	1.01	0.10	1.32	0.45	1.78	0.25	> 0.99	1.48	0.84	2.35	0.31	1.05	0.26	0.23
SMAD2	0.98	0.07	1.05	0.31	0.81	0.24	> 0.99	1.47	0.24	1.59	0.30	0.90	0.10	0.10
SMAD3	0.99	0.04	1.07	0.24	0.86	0.15	> 0.99	1.25	0.19	1.20	0.13	0.78	0.12	0.15
TGFB1	1.20	0.35	1.38	0.25	0.77	0.25	0.29	0.90	0.22	1.46	0.24	0.52	0.08	0.10
TGFB2	1.11	0.12	1.25	0.29	1.01	0.10	> 0.99	2.25	0.36	2.28	0.46	1.13	0.17	0.03*
VEGF	1.09	0.44	0.95	0.19	0.27	0.01	0.38	1.76	0.23	0.33	0.23	0.56	0.33	> 0.99
TET Enzymes														
TET1	1.02	0.07	1.30	0.37	0.86	0.17	0.66	2.06	0.22	2.09	0.31	0.95	0.24	<0.01**
TET2	1.09	0.42	0.98	0.06	1.10	0.27	> 0.99	2.49	1.00	3.29	1.34	1.96	0.77	0.99

Note:

mRNA levels of genes of interest in human airway smooth muscle (ASM) cells were measured by qPCR. Results are reported as mean values ± SEM, with 3-6 donors per group.

[A] Relative Expression Ratio (RER) values were calculated by the 2^{-ddCt} method, where the RER was set as 1.00 in the non-asthmatic lipofectamine (LF) group.

[B] 2-way ANOVA with Bonferroni's multiple comparisons tests were used to determine the p-value comparing siCTL and siTET1 groups in non-asthmatic ASMCs.

[C] 2-way ANOVA with Bonferroni's multiple comparisons tests were used to determine the p-value comparing siCTL and siTET1 groups in asthmatic ASMCs.

*$p<0.05$ and **$p<0.01$

DISCUSSION

In this study, we demonstrated that repeated exposures to common house allergens (HDM) in male mice increased the AHR and were associated with tissue/cell-specific increased 5hmC and Tet activity in the lung and isolated mouse ASMCs. We further showed the Tet1 up-regulation in accordance with the increased gene-specific hydroxymethylation of *Tgfβ2* (in both the mouse lungs and mouse ASMCs), *Smad2* (lung tissues only) and *Smad3* (MASMCs only), which consequently led to their increased gene expression levels (45). Given the fact that TET-mediated hydroxymethylation acts as the intermediate in the DNA demethylation pathway, we further examined the role of TET1 in allergic AHR in cells from both mouse and human lungs. By utilizing a *Tet1*$^{+/-}$ mouse model, we demonstrated that the diminished activity of Tet1 protects mice from HDM-induced AHR. Interestingly, comparing the WT and *Tet1*$^{+/-}$ mouse exposed to HDM the Tet1 deficiency had no effects on the HDM-induced neutrophilia, eosinophilia, and HDM-specific IgE production. We found that the Tet1 deficiency further increased the *Foxp3* in the lungs of the mice exposed to HDM, compared to the HDM-exposed WT mice, indicating that Tet1 depletion promotes the *Foxp3* transcription. FOXP3 (Forkhead box P3) is considered to be a master regulator for T-regulatory (Treg) cells (11, 84), and is often down-regulated in asthmatic patients, which showed increased *FOXP3* promoter DNA methylation associated with its gene suppression and activation of Treg cells (11) that alleviate the airway inflammation. Future studies on how Tet1 regulates *Foxp3* in mouse lungs could further improve the understanding of the epigenetic regulation of Treg cells in curing airway inflammation. Other than *Foxp3*, we did not find that Tet1 deficiency results in the reduction of Th2 cytokine (Il4, Il5, and Il13) production, and

postulated that the protection from the HDM-induced AHR from the Tet1 deficiency was not attributable to modulations in the Th2 inflammatory immune response. Previous findings have indicated that the pathophysiology of AHR can occur independently from the inflammatory immune response (26, 85, 86). There have also been extensive studies into the epigenetic mechanisms of the inflammatory immune response in asthma.

The current understandings of epigenetic regulation in asthma mainly focus on the immune response involved in chronic airway inflammation, which is a hallmark of asthma pathogenesis (42, 69, 70, 87-90). Naïve $CD4^+$ T cells differentiate into specific T helper cell subsets (Th1, Th2, Th17, and Treg), depending on the expression of the specific transcription factors (91). Asthma is generally considered to be a pro-inflammatory Th2-driven disease in which an overexpression of Th2 cells drives asthma pathogenesis by secreting the pro-inflammatory cytokines IL4, IL5, and IL13. IL4 works in a positive feedback loop to increase $CD4^+$ T cell differentiation into the Th2 lineage. Finally, the over-expression of Th2 cell activation exacerbates the risk of asthma by suppressing the production of the Th1 cells (70, 91). Therefore, the epigenetic regulation of $CD4^+$ T cell differentiation and T cell lineage maintenance in asthma has been widely studied. For example, Th2 cells express histone methylase SUV39H1, which trimethylates histone H3 on lysine 9 (H3K9me3) in key regulatory regions in Th1 genes, silencing the Th1 genes and maintaining the Th2 genes essential for Th2 differentiation and maintenance (91). Furthermore, the inhibition of SUV39H1 in an ovalbumin allergen induced asthma model showed an increased Th1 response and reduced allergic lung inflammation (91). These studies suggest that chromatin remodeling by histone

modification can skew the Th1/Th2 balance toward a Th2 allergic inflammation phenotype. In addition to histone modifications, DNA methylation has been found to regulate the Th1/Th2 imbalance in experimental asthma models.

An ovalbumin challenge increased the DNA methylation of the Ifng promoter, and was associated with an increased Th2 driven inflammatory immune response. The inhibition of DNA methylation with 5-aza-2-deoxycytidine resulted in the demethylation of the Ifng promoter, and protected the mice from ovalbumin induced airway inflammation (92). In the case of adult monozygotic twins, one with asthma and one without, the asthmatic twin showed impaired Treg activity that was associated with increased DNA methylation in the promoter region of *FOXP3*, and decreased *FOXP3* gene and protein expression (11). In addition, serum B-lymphocytes from HDM-allergic asthmatic subjects were globally hypermethylated, compared to those of the non-asthmatic controls (93). The HDM-allergic asthmatics also showed an increase in the IL4 receptor gene expression in B lymphocytes, which supports the idea of increased IL4 signaling and the B lymphocyte activation implicated in the immune dysregulation of asthma (94). There have been extensive studies into the epigenetic mechanisms of the inflammatory immune response in asthma, because they can be performed on the easily accessible inflammatory cells. Our results indicated that the epigenetic regulation of airway reactivity by Tet1 occurs mainly in the ASM cells. This may support the previous findings that the pathophysiology of AHR can occur independently from the inflammatory immune response (26, 85, 86).

We demonstrated that TET1 plays an important role in global and gene-specific methylation changes, specifically in ASM cells. Given the fact that epigenetic changes are cell and tissue-specific, different tissues might have unique 5mC and 5hmC profiles that can be altered through the induction of TET activity (52, 95) upon various exposures to environmental pollutants or chemicals. Our findings indicate that HDM challenges may induce TET1 up-regulation and its mediated DNA hydroxymethylation, specifically in ASMCs, and alter cell functions through the epigenetic gene regulation of ASM phenotypic genes. In addition to inflammatory cells, the ASMCs lining the airway lumen are largely implicated in asthma pathogenesis through aberrant ASM function, structure, or inflammatory response, which result in the increased wheezing and shortness of breath associated with asthma (85). Aberrant ASM functions, such as increased contraction or bronchoconstriction, and decreased relaxation contribute to airway narrowing, while increased ASM mass, basement membrane thickening, and mucus gland hyperplasia are implicated in the structural changes of airway remodeling and ASM synthesis. The release of IL5, IL13, and TGFβ cytokines in ASMCs further propels airway inflammation (85, 96-98). Our findings reiterate that the epigenetic role of TET1-mediated DNA hydroxymethylation as a cell-specific response to environmental stimuli is able to modulate the development of asthma phenotypes. Thus, there is great potential for the design of epigenetic therapies targeted toward TET1-mediated asthma pathogenesis (69, 99). Traditionally, beta-agonists are the most commonly used therapy for treating asthma, by which the beta2-adrenergic receptor (β2-AR) is activated to increase cyclic adenosine monophosphate (cAMP) production, which activates protein kinase A (PKA), and subsequently relaxes the ASM through the inhibition of myosin light chain kinase

(MLCK) (97). Nevertheless, the downside to therapy using beta-agonists for ASM relaxation is that prolonged use can lead to tachyphylaxis, or the loss of β2-AR expression that causes desensitization to the agonist and inability to control bronchoconstriction. Our data demonstrated a novel role for the specific 5mC dioxygenase, TET1, in regulating ASM function *in vitro* and in the development of allergen-driven AHR *in vivo*. It provides considerable new insights into the role of the ASM in the development of AHR, and the development of modulators of this critical pathway to ameliorate allergen-induced AHR and asthma.

We were able to translate our experimental asthma model to human asthmatic ASM cells by demonstrating that TET1 up-regulation is a generalized phenomenon in ASMCs for individuals with asthma. A heterogeneous population of lung donors with unknown exposures or allergic profiles may expect inter-individual epigenetic variations. Nevertheless, we were able to confirm the generalized role of increased 5hmC, TET1 expression, and TET activity in ASMCs from individuals with asthma when compared to non-asthmatics. There were discrepancies between the 5mC levels, which were unchanged in the MASMCs of the HDM-exposed mice, but increased in human asthmatic ASMCs. The differences in the global 5mC levels comparing mouse and human ASMCs could be due to species differences in the overall CpG contents as well as disparate exposures over varying periods; with human asthmatic ASMCs exposed to complex mixtures of environmental agents over decades of disease progression. Despite these differences, the knockdown of *TET1* by si*TET1* in human asthmatic ASMCs could modulate the aberrant ASM phenotypic gene expression and cell proliferation, similar to

the loss of Tet activity upon the regulation of ASM genes and AHR in our *Tet1*$^{+/-}$ mouse model. To our knowledge, this study is the first to investigate the role of Tet1-mediated DNA hydroxymethylation associated with altered AHR phenotypes. Given the reversible nature of epigenetic changes in the cells, our data may provide insights into the development of modulators of this critical pathway to ameliorate allergen-induced AHR and asthma.

There is great potential for the design of epigenetic therapies targeted toward TET1-mediated asthma pathogenesis, particularly in regulating ASM functions. Traditionally, beta-agonists are the most commonly used medication for treating asthma by activating the beta2-adrenergic receptor (β2-AR) to increase cyclic adenosine monophosphate (cAMP) production, which in turn activates protein kinase A (PKA) and subsequently relaxes the ASM through the inhibition of myosin light chain kinase (MLCK) (97). Nevertheless, the downside to using beta-agonists for ASM relaxation is that prolonged use can lead to tachyphylaxis, or the loss of β2-AR expression that causes desensitization to the agonist and inability to control bronchoconstriction (83, 97). Moreover, epigenetic regulation of ASMCs has been shown to have therapeutic potential in asthma treatment (42, 44, 100, 101). Recently, we have demonstrated that the suppression of *PDE4D* gene expression in asthmatic ASMCs, through CpG site specific methylation at the *PDE4D* promoter with methylated oligonucleotides, could abrogate the ASMC phenotypes seen in asthmatics (102). Additionally, the administration of the histone deacetylase inhibitor, valproic acid, was shown to protect mice from ovalbumin-

induced AHR and airway remodeling, indicating the potential for histone modulators in the treatment of asthma (66).

There are limitations in our current study. First, we have not presented the role of TET1 in airway remodeling commonly seen human asthmatics, because our HDM-induced AHR mouse model only demonstrated the acute effects of allergens on airway reactivity and inflammation. To address these concerns in the future, the use of chronic HDM exposure upon the Tet1 deficient mice may better capitulate the effects of lifelong exposure to allergens and AHR. We speculate that chronic repeated exposures to HDM could further exacerbate the HDM-driven AHR by modulating ASM remodeling via aberrant epigenetic changes in the ASM cells, at least partly through TET-mediated hydroxymethylation. Second, *Tet1* homozygous mice ($Tet1^{-/-}$) were not employed in our AHR model. Previous investigations revealed that the embryonic loss of Tet1 is not lethal, but that it produces $Tet1^{-/-}$ offspring with significantly reduced body size, weight, and fertility (79). Therefore, due to the fertility limitations and runt phenotypes, $Tet1^{-/-}$ were less abundant and more susceptible to pre-mature death, which is why we opted to compare the effects of acute HDM between $Tet1^{+/+}$ and $Tet1^{+/-}$ mice. Despite the use of $Tet1^{+/-}$ mice, the loss of only one allele was adequate to see significant phenotypic changes in the lung function. Therefore, we would expect that chronic HDM exposure would further increase the HDM-induced airway resistance, measured by AHR, and that the deficiency in Tet1 ($Tet1^{+/-}$) would create a larger reduction in the HDM-induced AHR, and an even more substantial reduction with the complete loss of Tet1 ($Tet1^{-/-}$), given that the mice survived the chronic HDM challenge regimen. In addition, our mouse

model used a whole body knockout of *Tet1*, which could have unforeseen implications upon our HDM-induced AHR findings in the lung and isolated MASMCs.

Tet1 suppression does not acutely regulate allergen-driven IgE or eosinophilia, suggesting that Tet1 deficiency may not influence the acute airway inflammation response; however, Tet1 may play a role in chronic airway inflammation and the development of AHR. To further the understanding of the role of Tet1 depletion in immune cells, epithelial cells, and ASM cells upon HDM-induced AHR, tissue-specific knockout studies of Tet1 could be employed. Furthermore, it is questionable whether Tet1 up-regulation only occurs upon HDM exposure in the mouse. However, this is unlikely because we demonstrated that TET1 up-regulation is a generalized phenomenon seen in human asthmatics. This is an ideal illustration of chronic and varied environmental exposures leading to increased AHR. We can postulate that the effects of allergens on Tet1 in our mouse model will also been seen with other allergens or environmental exposures. Future studies can test whether TET1 up-regulation in human asthmatics is influenced by environmental exposures, allergen sensitivities, severity of disease, or medication use in a well-defined human asthmatic population.

In summary, our results provide a novel perspective of the epigenetic regulation of allergen-driven changes in ASM function and AHR. We directly address gaps in our understanding of how environmental insults may initiate asthma. These findings also introduce the novel concept that TET1 modulators might induce bronchodilation or reduce asthmatic phenotypes in humans.

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CHAPTER 3

THE ROLE OF OXIDATIVE STRESS UPON TET1 REGULATION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

ABSTRACT

The significance of DNA methylation in modulating gene expression changes and biological mechanisms has been implicated in many diseases. Currently, oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) enzymes, considered an intermediate in the DNA demethylation pathway, has renovated previous concepts of epigenetic regulation. Previously, we demonstrated that *Tet1* deficiency protected mice from allergen-induced airway hyperresponsiveness, a cardinal feature of asthma. Further, we demonstrated that *TET1* up-regulation contributed to the aberrant airway smooth muscle cell (ASMC) phenotypes seen in the clinical features of asthma. Therefore, by understanding the mechanisms of *TET1* regulation in human airway smooth muscle cells (ASMCs), we can gain insight into the aberrant ASMC phenotypes implicated in the pathological outcomes of asthma. Changes in oxidant balance are proposed to trigger the production of alpha-ketoglutarate (alpha-KG), which can activate TET enzymes and potentially disrupt cellular and biological processes. We aimed to investigate if oxidant balance modulates the regulation of TET enzymes, especially *TET1*, in the ASMCs from non-asthmatic and asthmatic patients. We found that transforming growth factor, beta 1 (TGFβ1) increased oxidative stress in ASMCs, which in turn increased citrate metabolism gene *IDH2* and alpha-KG production, as well as up-regulated TET activity and cell proliferation in non-asthmatic cells. By contrast, Sulforaphane (SFN) treatment abrogated oxidative stress levels and reduced alpha-KG metabolite levels that corresponded with decreased TET activity in both non-asthmatic and asthmatic cells. Furthermore, SFN reduced cell proliferation phenotypes and cell proliferation genes (*PCNA, CCND1*, and *TGFβ2*) in addition to cell

contraction genes (*MYLK2* and *CAMK2D*) in both non-asthmatic and asthmatic ASMCs. In summary, we demonstrated that modulation of oxidant balance can alter the generation of alpha-KG metabolite pools and subsequently regulate TET activity and ASM cell phenotypes. Our findings suggest a therapeutic potential of anti-oxidants to modulate aberrant *TET1* activity in ASMCs thereby ameliorating the abnormal phenotypes seen in asthmatics.

INTRODUCTION

The ability of DNA methylation, the most commonly studied epigenetic mechanism, to modify gene regulation and biological mechanisms generated considerable research interest on the DNA methylation patterns associated with disease mechanisms. The discovery of ten-eleven translocation (TET) proteins, which alter DNA methylation profiles by catalyzing the oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), an intermediate in the DNA demethylation pathway, has completely changed the epigenetic landscape. Therefore, understanding the DNA methylation machinery involved in DNA hydroxymethylation profiles, in addition to DNA methylation changes, gives considerable insight into the epigenetic modifications possibly implicated in disease mechanisms. For example, in Alzheimer's disease patients, post-mortem hippocampus brain tissue exhibited decreased 5hmC content as the disease pathology advanced with increased hippocampal amyloid plaques and neurofibrillary tangles (103). In melanoma cancers, the 5hmC content decreased depending on the progression of the tumor with high 5hmC content in benign melanocytic nevi and a significant decrease in primary and metastatic melanomas that correlated with decreased *TET1, TET2*, and *TET3* gene expression compared with the nevus control (104). Aberrant

DNA hydroxymethylation has been shown to influence asthma pathogenesis (42). In Chapter 2, we demonstrated in an allergen-induced model of airway hyperresponsiveness (AHR) that the loss of *TET1* decreased 5hmC levels and was associated with decreased AHR in mouse lungs. Furthermore, we also reported that TET activity and its mediated hydroxymethylation could alter changes in ASM phenotypic genes in both mouse and human ASMCs.

The co-factors involved in DNA hydroxymethylation are thought to be critical to the activation of TET proteins. TET proteins are 2-oxoglutarate-dependent dioxygenases that require co-factors, such as iron(II) (Fe^{2+}), oxygen, and 2-oxoglutarate (alpha-KG), for the oxidation of 5mC into 5hmC. Recent evidence has supported the idea of TET activity enhancement through ascorbic acid (AA) or vitamin C (105-107). AA can uniquely interact with the C-terminal catalytic domain of TET enzymes to promote their folding and/or recycling of the cofactor Fe^{2+}(105). Aside from Fe^{2+}, alpha-KG is another co-factor that regulates TET activity. In the first step of the citric acid cycle, citrate synthase combines acetyl-coA with oxaloacetate for the irreversible formation of citrate, which is converted to isocitrate by the aconitase enzyme (108). Isocitrate dehydrogenase enzymes (IDH1, IDH2, and IDH3) subsequently convert isocitrate into alpha-KG, an intermediate in the citric acid cycle and the required co-factor for TET activity (109). In mouse embryonic stem cells, the addition of cell-permeable alpha-KG in the medium increased intracellular alpha-KG levels and decreased overall DNA methylation profiles; it is also associated with the increased expression of inner cell mass and germline associated genes (*Wdfc15a, Asz1,* and *Dazl*) (110). These results indicate that increased alpha-KG metabolites up-regulate TET-mediated DNA demethylation pathways to

decrease global DNA methylation, as well as the gene-specific hypomethylation of *Wdfc15a, Asz1*, and *Dazl* to increase gene expression. In a zebrafish melanoma model, the overexpression of *IDH2* increased 5hmC content and prolonged the tumor-free survival of zebrafish with small and less invasive tumors compared with the control (104). The overexpression of IDH2 activity increased the generation of alpha-KG and up-regulated the TET-mediated 5hmC content, which protected zebrafish from aggressive melanocytic metastasis and death. Imbalances in the oxidant and antioxidant homeostasis have been hypothesized to modify the production of alpha-KG. Under high oxidative stress conditions, increased nicotinamide adenine nucleotide (NAD) levels in the mitochondria activate sirtuin NAD^+ -dependent deacetylases (SIRT) (111). Activation of SIRT3 results in the deacetylation of IDHs, which in turn increases IDH1 and IDH2 activity, as well as the subsequent activation of citrate metabolism for increased alpha-KG production (112, 113), which facilitates the activation of TET. For example, the *in vivo* administration of glucose in mice exhibited increased alpha-KG metabolite levels in liver tissue that corresponded with increased TET-mediated 5hmC content in the liver (114). Therefore, oxidative stress imbalances can affect citrate metabolism by altering SIRT and IDH activity, and it also affects the production of alpha-KG metabolite levels that are sensitively linked to the regulation of TET activity.

Increased oxidative stress has been shown to underlie the disease mechanisms among asthmatic patients (14, 115-117). Furthermore, chronic exposures to air pollution, pollen, or environmental tobacco smoke have been shown to increase reactive oxygen species (ROS) production, which plays a role in the induction of airway inflammation and remodeling in the lungs (118-120). In the airways, a relentless and complex

interaction of oxidative stress occurs from external sources inhaled through the environment and internal sources from the inflammatory immune response (115). Although the airways are under assault with every breath, many anti-oxidant mechanisms mitigate the ROS imbalances in the airways. Exposures to air pollution, pollen, or environmental tobacco smoke result in inflammatory cell recruitment, activation of NADPH oxidases, and epithelial cell mitochondrial dysfunction, which generate the toxic anion superoxide ($O_2^{\cdot -}$) that can be neutralized by scavenger super oxide dismutase enzymes into hydrogen peroxide (H_2O_2) and oxygen (O_2) (115, 121). In peroxisomes, catalase degrades H_2O_2 into water (H_2O) and oxygen (O_2). Also, glutathione peroxidase degrades H_2O_2 into water by reducing glutathione (GSH) into oxidized glutathione-disulfide, which can be converted back to GSH by glutathione reductase. At any step along this process, any residual or excess superoxide and H_2O_2 can interact with macromolecules (DNA, lipids, protein) in the cell to exhibit oxidative damage and inflict airway oxidative stress. Substantial increases in ROS production may damage the airway epithelium to facilitate the increased presentation of allergen/pathogenic substances to airway dendritic cells and other inflammatory cells and further activate the secretion of pro-inflammatory cytokines and chemokines (122). These inflammatory mediators can further modulate the functions of the resident cells in the airway and lead to the development of airway remodeling.

Taken together, strong evidence indicates that an imbalance in redox cycling fosters increased airway oxidative stress in asthma. Herein, we hypothesize that increased ROS production in the lungs facilitates increased alpha-KG production and the subsequent TET activation and then ultimately modulates lung function. We already

demonstrated the significant role of *TET1* in human ASMCs in Chapter 2, so we now investigate if altered intracellular ROS levels can trigger citrate metabolism changes to modulate alpha-KG production and TET activity in human ASMCs. Furthermore, we will determine whether this proposed mechanism can reverse the aberrant cell phenotypes seen in asthmatics.

METHODS

Cell culture

Human ASM cells were derived from the tracheas of deceased non-asthmatic and asthmatic donors from the National Disease Research Interchange (Philadelphia, PA), as previously described (83). Donor characteristics (age, gender, race, history) are described in Table 1. Cells were received at passage 3 and maintained in DMEM/F-12 medium (Corning Cellgro, Manassas, VA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 1% Penicillin-Streptomyocin Solution (Corning Cellgro, Manassas, VA), and 1% L-Glutamine (Corning Cellgro, Manassas, VA). Cells were not used once they reached passage 12.

Drugs treatment

Human ASM cells were treated with either 30 μM DL-Sulforaphane (SFN) (Sigma-Aldrich, St. Louis, MO) or 1 ng/mL human TGFβ1 (R&D Systems, Minneapolis, MN) for the desired time point.

Measurement of reactive oxygen species (ROS) production

Human ASM cells were grown in 96-well black plates for 2 days before the cells were incubated with 50 μM of 2',7'- Dichhlorofluorescein diacetate (DCFDA) (Sigma Aldrich, St. Louis, MO) for 1 hour. After DCFDA incubation, the cells were treated with either 30

μM SFN, 1 ng/mL TGFβ1, or a range of hydrogen peroxide (0–1000 μM as a positive control to measure ROS production). At the desired time points (3, 6, and 24 hours), ROS was detected by the formation of 2',7'- Dichhlorofluorescein to measure ROS production (425 nm/emission 530 nm).

Cell viability measurement

Trypan Blue Solution (Corning Cellgro, Manassas, VA) was used to distinguish viable and non-viable cells after drug treatment.

Cell proliferation measurement

At a desired time after drug treatment, the cells were incubated with 5-bromo-2'-deoxyuridine (BrdU) solution for 6 hours, according to the BrdU Cell Proliferation Assay Kit (Cell Signaling, Beverly, MA). Briefly, BrdU analog incorporation into DNA was fixed and denatured before the BrdU detection antibody was added for 1 hour. Secondary HRP-linked antibody was added and incubated for 0.5 hour. Tetramethylbenzidine substrate was added to develop color. Color formation was measured at an absorbance of 450 nm.

Assessment of mRNA level

Total RNA (1 μg) isolated from human ASM cells by TRIzol (Invitrogen, Carlsbad, CA) was reverse transcribed with iScript Reverse Transcriptase (Bio-Rad, Hercules, CA). Gene transcript levels were quantified by SYBR Green-based or TaqMan-based real-time PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of transcripts normalized to *RPL19*. The primer sequences used for qPCR are listed in Table 1.

Alpha- Ketoglutarate (alpha-KG) assay

At a desired time after drug treatment, the cells were collected, and the cell number was counted. With the use of a Dounce homogenizer on ice cold alpha-KG assay buffer, $3x10^{\wedge 6}$ cells were homogenized before the samples were processed according to the alpha-KG Assay Kit (Sigma Aldrich, St. Louis, MO). The colorimetric detection of alpha-KG at 570 nm was plotted against a standard curve to determine the alpha-KG concentration.

Assessment of TET activity

Nuclear protein extracts were isolated from cells with the use of EpiQuick Nuclear Extraction Kit (Epigentek, Farmingdale, NY). Proteins were quantified for total protein levels (Pierce BCA Protein Assay Kit, ThermoScientific, Rockford, IL). To measure TET activity, 2–6 μg of total nuclear protein was used (Epigentek, Farmingdale, NY). TET activity was assayed with the use of Epigenase 5mC Hydroxylase TET Activity/Inhibition Kit (Epigentek, Farmingdale, NY). In brief, TET substrate was added to all wells and incubated at 37 °C for 1.5 hours. Then, nuclear proteins were incubated with TET substrate at 37 °C for 1.5 hours to convert the TET substrate into 5hmC product. Capture antibody was added to recognize the TET-converted products, and then detection antibody was added. Finally, the colorimetric conversion of the developer solution was measured at 405/655 nm, and TET activity was calculated compared with standards.

Sulforaphane (SFN) exposure in mice and AHR measurement

Adult male C57BL/6J mice, 6 to 8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed under controlled lighting (12 hours light and 12 hours dark) and temperature (21–22 °C) conditions. The mice were housed in

polysulfone-ventilated cages (Technoplast, Exton, PA) and provided with Harlan's Teklad Global 18% Protein Extruded Rodent Diet 2018SX and fresh water ad libitum. Male mice, at 6 to 8 weeks of age, were sensitized on day 0 (treatment day) with 50 μg of house dust mite (HDM) extract (*D.Pteronyssinus*, Greer Laboratories, Lenoir, NC) (diluted in 200 μL PBS) or saline (PBS) intraperitoneally (i.p.). Two weeks after (day 14), the mice were challenged with 50 μg of HDM (diluted in 50 μL PBS) or PBS (intratracheally, i.t.) twice on days 14 and 21. Another group of mice was exposed to 50 mg of SFN (diluted in 200 μL PBS) i.p. on days 1, 3, and 5 after the first HDM sensitization. The mice were further challenged by HDM i.t. twice on days 14 and 21. Pulmonary function test for AHR was measured on day 23. The SFN and HDM exposure protocol is depicted in a schematic in Figure 7. The mice were anesthetized i.p. with pharmaceutical-grade ketamine and xylazine. After approximately 15 minutes, a 20-gauge IV catheter was placed in the trachea and secured with a suture. The mice were maintained on a mechanical ventilator that delivers a constant inspiratory flow of air. The mice were administrated with increasing doses of aerosolized Methacholine chloride (Mch) (Sigma-Aldrich, St. Louis, MO) (0.1, 0.3, 1, 3, 10, and 30 mg/mL) while maintained on the ventilator. Mch challenge was followed by airway resistance measurement by flexiVent (SCIREQ, Montreal, Canada). AHR was assessed as the change in pulmonary resistance (R cm/H2O/mL/s) compared with the baseline after the Mch challenge. Lung tissues were harvested and subject for alpha-KG and TET activity assays, as described.

Statistical analysis

Results were expressed as mean ± standard error mean. Student t-test was used to analyze the differences in ASM characteristics between non-asthmatic and asthmatic ASM cells. For group comparison, two-way ANOVA of multiple comparisons with Bonferroni's and Tukey's corrected tests was applied to determine if the data between treatment groups was statistically significant. The details of the statistical analysis are described in each data figure and table. All data were analyzed and plotted with Prism6 (GraphPad, La Jolla, CA).

Study approval

All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD) (MO14H241). Human ASMCs from non-asthmatics and asthmatics were either purchased from a commercial vendor (Lonza, Gaithersburg, MD) or obtained from deceased donors in accordance with the Institutional Review Board of the University of Pennsylvania and Johns Hopkins University.

Table 1. Sequences of primers used for qPCR.

Primers for qPCR for human genes.

Primer Name	Sequence (5' to 3')
hADAM33-F	ACC TAG AAT GGT GTG CCA GA
hADAM33-R	GCA CAG TGG CAG TTA TGG TTG
hAID-F	TGG ACA GCC TCT TGA TGA ACC
hAID-R	GTA GCA CTG TCA CGC CTC TT
hCAMK2D	IDT Primetime Hs.PT.56a.27233732
hCCND1	IDT Primetime Hs.PT.56a.3857509
hCOL3A-F	GTT GCA CGA AAC ACA CTG GG
hCOL3A-R	AAA AGC AAA CAG GGC CAA CG
hDNMT1-F	GAT CGA GAC CAC GGT TCC TC
hDNMT1-R	CGG CCT CGT CAT AAC TCT CC
hIDH1-F	TACTTCAGAAGCGGAGGCAC
hIDH1-R	TAGTTTATCGCCTGCCGGG
hIDH2-F	GCTCTCCAGCTTGGGATGG
hIDH2-R	TCCTTTTGTCGGCATAGTGGC
hIL1B-F	GGC TGC TCT GGG ATT CTC TT
hIL1B-R	AGT CAT CCT CAT TGC CAC TGT AA
hIL6-F	CAG CCC TGA GAA AGG AGA CAT
hIL6-R	GGT TCA GGT TGT TTT CTG CCA
hMBD1-F	CAG CCT GCC AGG TAA CAG AA
hMBD1-R	CCT CGG CTC CTT TCC ACAAT
hMBD4-F	CAC CTC CTC GGT CAC CTT TT
hMBD4-R	CCA TTT TGC CTG AGG TCC GA
hMECP2-F	ACT CCC CAG AAT ACA CCT TGC
hMECP2-R	TTC TTC CCT GAG CCC TAA CAT C
hMYLK2	IDT Primetime Hs.PT.56a.27638038
hPCNA-F	TCC CTT ACG CAA GTC TCA GC
hPCNA-R	AGT CTA GCT GGT TTC GGC TT
hSIRT1-F	TTGGGTACCGAGATAACCTTCTG
hSIRT1-R	ATGGACCTATCCGTGGCCTT
hSIRT3-F	TTGCAGAGGCCTCCCAGAC
hSIRT3-R	GACCTTCTTCCACCTTTAATACTCG
hTDG-F	CTT GCC CAA GAG GAT GCA AAG
hTDG-R	AGG TTC ACT GCT GCA TGG AT
hTET1-F	CGA GTT GGA AAG TTT GCC CG
hTET1-R	CAC AAG GTT TTG GTC GCT GG
hTET2-F	TGG CTG CCC TTT AGG ATT TGT
hTET2-R	GAA TGT TTG CCA GCC TCG TTC
hTGFB1-F	CGG CCT TTC CTG CTT CTC AT
hTGFB1-R	TCT TCT CCG TGG AGC TGA AG
hTGFB2-F	TTC TTC CCC TCC GAA ACT GTC
hTGFB2-R	GGT CTG TAG AAA GTG GGC GG

RESULTS

Oxidant levels in ASMCs can modulate ASM cell phenotypes

We demonstrated the role of increased oxidative stress in asthmatic ASMCs, which is well reported in asthmatic patients (122, 123). All ASMC donors were adults, with an even mix of males and females, although most were Caucasian (Table 2). Examining their history, we found that both non-asthmatics and asthmatics generally reported no tobacco use, and most non-asthmatics died from head traumas, whereas most asthmatic ASMC donors died from a fatal asthma attack (Table 2). Given the heterogeneous ASMC donor characteristics, we first determined if asthmatic ASMCs had higher basal ROS levels than the non-asthmatics. ROS level was measured by the production of DCFDA, an indicator of oxidative stress, in cells over 24 hours (Figure 1). A significant increase in ROS levels in asthmatic ASMCs was evident compared with non-asthmatics at 3 and 6 hours, although not statistically significant at 24 hours (Figure 1). We observed that the ROS level at 24 hours was approximately double the level at 6 hours in both non-asthmatic and asthmatic ASMCs (Figure 1). Overall asthmatic ASMCs trended towards higher ROS levels than non-asthmatics. Together, our results indicate that at baseline, the ROS levels in ASMCs are significantly increased in asthmatics.

Table 2. Characteristics of non-asthmatic and asthmatic ASM cell donors.

Label ID	AGE	GENDER	RACE	HISTORY
Non-asthmatic Airway Smooth Muscle (ASM) Cells				
NO1	18	Female	Caucasian	Head trauma, smoked 1/3 pack per day for 4 months
NO2	17	Male	Caucasian	Gunshot wound to head, smoked unknown amount for 3 days a week
NO3	24	Female	Caucasian	Cardiac arrest, no tobacco use
NO4	31	Female	Black	Aneurysm, tobacco use for 10 years
NO8	50	Female	Caucasian	Head trauma, no tobacco use
NO10	21	Male	Caucasian	Head trauma, no smoking
NO11	11	Male	Caucasian	Head trauma, no tobacco use
NO12	50	Female	Caucasian	Head trauma, no tobacco
NO13	17	Male	Black	Gunshot wound to head, no tobacco use
NO14	48	Male	Hispanic	Cerebrovascular accident, no tobacco use
NO15	22	Male	Black	Blunt injury, no tobacco use
Label ID	**AGE**	**GENDER**	**RACE**	**HISTORY**
Asthmatic Airway Smooth Muscle (ASM) Cells				
AS1	18	Female	Caucasian	Anoxia, asthma attack, no tobacco use
AS2	14	Male	Caucasian	Anoxia, asthma attack, no tobacco use
AS3	27	Female	Caucasian	Anoxia, asthma attack, tobacco use 5 times day for 7 years; Quit 2 months ago
AS4	25	Female	Caucasian	Cardiac arrest, asthma attack, smoked 1/2 pack per day for more than 5 years
AS5	26	Female	Black	Asthma attack, anoxia, no tobacco or drug use
AS6	38	Male	Caucasian	Anoxia, asthma attack, no tobacco use
AS7	35	Female	Caucasian	Anoxia, no tobacco or drug use
AS10	54	Male	Caucasian	Anoxia, asthma attack, no tobacco use
AS11	46	Female	Caucasian	Asthma attack, anoxia, no tobacco use

Note:
All human ASM cells were derived from deceased tracheas of asthmatic and non-asthmatic donors from the National Disease Research Interchange (Philadelphia, PA), as previously described (*Robinett et al. 2014*).

Fig 1

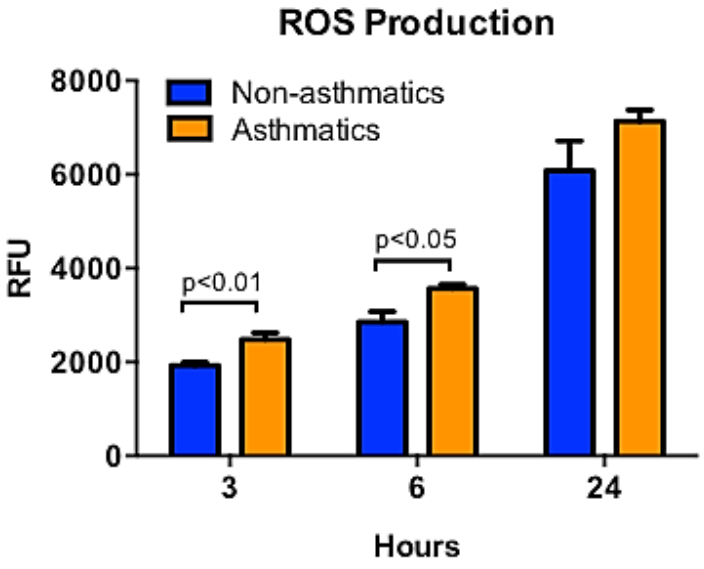

Figure 1. Asthmatic ASMCs have increased ROS production. Basal levels of non-asthmatic and asthmatic ROS levels were measured by the relative fluorescence units (RFU) measured at Excitation 425nm/Emission 530nm in the DCFDA assay at 3, 6 and 24 hours. Results expressed as mean values ± SEM, with 5 non-asthmatic and 5 asthmatic donors per group. Multiple t-test comparisons were used to determine the p-value and statistically significant comparisons were noted.

To test the effects of oxidant balance alterations on ASMCs, we exposed ASMCs to TGFβ1 or SFN and examined the phenotypic changes of non-asthmatic and asthmatic cells. In all the subsequent experiments on ASMCs, the data for each ASMC donor were calculated as a ratio compared with the average of the non-asthmatic control (untreated) group. The average of the non-asthmatic control was set at 100%. We decided to express the data in this manner, so we could detect the significant changes in each assay, which can be obscured if expressed as raw values because of the baseline noise attributed to the heterogeneity of the non-asthmatic and asthmatic donors. For example, at 24 hours, a 25% increase in ROS production was observed in asthmatic ASMCs compared with non-asthmatics (Figure 2A). Similarly, at 3 hours, asthmatic ASMCs had ~40% more ROS than non-asthmatic ASMCs (Figure 2B). These results confirm our previous findings of increased basal ROS level in asthmatic ASMCs (Figure 1). TGFβ1 is a pleiotropic cytokine that plays many roles in the lung through complex TGFβ signaling pathways that have effects on cell apoptosis, proliferation, and angiogenesis. The TGFβ pathway also influences biological processes in endothelial cells, goblet cells, fibroblasts, and ASMCs (124, 125), and ultimately contribute to the development of airway remodeling. Treatment with TGFβ1 increased ROS levels in both non-asthmatic and asthmatic cells by 25% and 10%, respectively (Figure 2A). Notably, TGFβ1 treatment induced the ROS levels in non-asthmatic cells to the ROS levels shown in the asthmatic controls. In contrast, treatment with the antioxidant SFN for 3 hours decreased the ROS levels by 30% in asthmatic ASMCs, but it had no effect in non-asthmatic cells (Figure 2B). These results indicated that the ROS levels in ASMCs could be modulated by TGFβ1 and SFN.

Fig 2

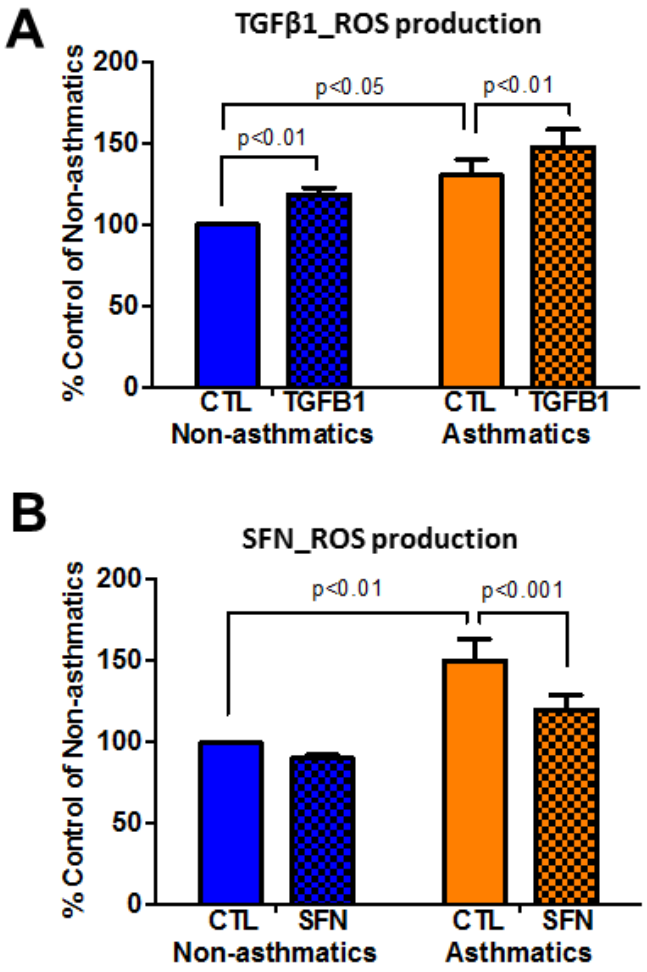

Figure 2. TGFβ1 increased ROS production and SFN decreased ROS production. DCFDA assay was used to measure ROS production after **(A)** treatment with TGFβ1 at 1ng/mL for 24 hours and **(B)** with Sulforaphane (SFN) at 30uM for 3 hours. Relative change of ROS production in each group was normalized to the average of the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 4 non-asthmatic and 5 asthmatic donors per group. Two-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted.

Next, we sought to determine if the modification of ROS levels corresponded to alterations in ASMC phenotypes, such as cell viability and cell proliferation (Figure 3). At baseline, the number of viable cells between non-asthmatic and asthmatic ASMCs was not different (Figure 3A and B, left panel). But at baseline, measurement of the BrdU incorporation in cells indicated that asthmatic ASMCs showed increased cell proliferation (>50%) compared with non-asthmatics (Figure 3A and B, right panel). This finding suggests that asthmatic ASMCs are more proliferative than non-asthmatic cells, a characteristic that is commonly demonstrated in asthmatic patients (98). TGFβ1 caused a non-statistically significant increase ($p=0.06$) in the number of viable cells in non-asthmatics but not in the asthmatic group (Figure 3A, left panel). Using the BrdU assay, we demonstrated that TGFβ1 increased the cell proliferation of non-asthmatic ASMCs by 30% (Figure 3A, right panel). The result suggests that TGFβ1 treatment in non-asthmatic ASMCs not only increased the number of viable cells but also drove them toward a proliferative phenotype. Surprisingly, TGFβ1 slightly decreased the cell proliferation of asthmatic ASMCs by 20%, as measured by the BrdU assay. On the other hand, exposure to SFN decreased both the number of viable cells and cell proliferation in both non-asthmatic and asthmatic ASMCs (Figure 3B). SFN decreased non-asthmatic ASM cell number and cell proliferation by 30% and 70%, respectively (Figure 3B). In asthmatic ASMCs, SFN decreased the cell number by 50% and reduced cell proliferation by 140% (Figure 3B). The reduction of cell proliferation by SFN in asthmatic ASMCs was larger than that in non-asthmatic cells, and indicates that SFN has a very compelling anti-proliferative property in ASMCs from patients with asthma. SFN is a potent inducer of nuclear factor erythroid-2 related factor-2 (NRF2) activation, which is a critical

transcription factor for antioxidant response elements located in the promoter of anti-oxidative stress and phase II detoxifying genes (126). In asthmatic ASMCs, NRF2 activity is usually at a very low level (30), and we may speculate that SFN exposure may boost up NRF2 activity and induce a dramatic increase in the transcription of genes related to cell proliferation. However, future experiments are required to prove this hypothesis.

Fig 3

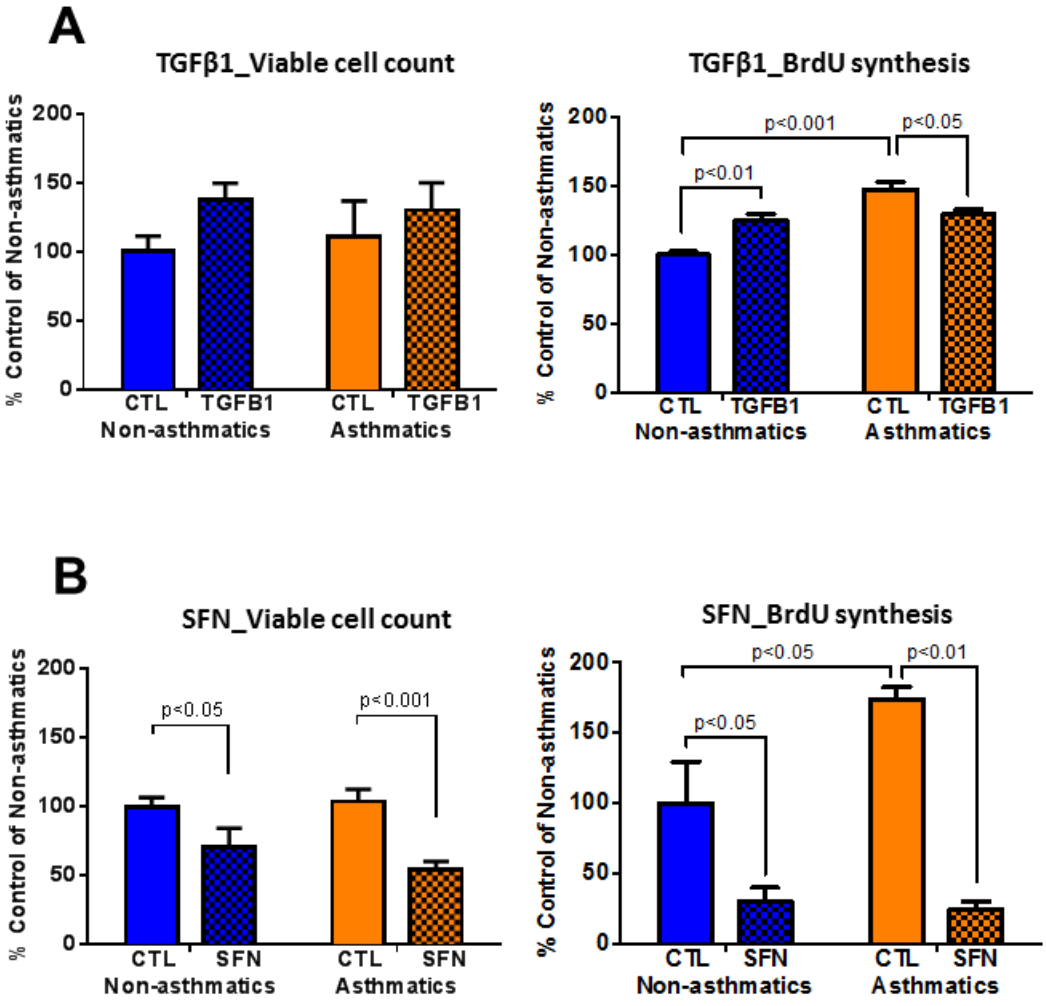

Figure 3. TGFβ1 increased cell proliferation and SFN decreased cell proliferation. BrdU incorporation and Trypan Blue cell viability assays were used to examine the cell proliferation and cell viability after **(A)** treatment with TGFβ1 at 1ng/mL for 48 hours and **(B)** with Sulforaphane (SFN) at 30uM for 24 hours (BrdU incorporation assay) or 48 hours (Trypan Blue cell viability assay). Relative changes in BrdU incorporation and cell viability were normalized to the average of the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM. BrdU incorporation on TGFβ1 treatment, 4 non-asthmatic and 4 asthmatic donors. Trypan Blue cell viability assay on TGFβ1 treatment, 3 non-asthmatic and 3 asthmatic donors. BrdU incorporation on SFN treatment, 4 non-asthmatic and 2 asthmatic donors. Trypan Blue cell viability assay on SFN treatment, 3 non-asthmatic and 6 asthmatic donors. Two-way ANOVA with Bonferroni's multiple comparisons tests were used and statistically significant comparisons were noted.

In addition to the increased cell proliferation, increased cell contraction, synthesis of collagen, and growth factors were measured since they are considered to be aberrant ASMC phenotypes contributing to airway remodeling and AHR. We measured the changes in mRNA level of ASM phenotypic genes (cell proliferation, *CCND1* and *PCNA*; collagen synthesis, *COL3A*; cell contraction, *CAMK2D* and *MYLK2*; and growth factors/mediators, *ADAM33* and *TGFβ2*) after 48 hours of TGFβ1 (Table 3) and SFN (Table 4) treatment. Treatment of TGFβ1 did not change the expression of *CCND1* and *PCNA* in non-asthmatic or asthmatic ASMCs (Table 3). However, TGFβ1 increased the gene expression of *COL3A* by 100% in non-asthmatic ASMCs (Table 3). Asthmatic ASMCs also showed a 70% increase in *COL3A* expression with TGFβ1 treatment (Table 3). This finding suggests that TGFβ1 may facilitate collagen synthesis in ASMCs. Exposure to TGFβ1 caused a three-fold increase in *MYLK2* expression in non-asthmatic cells and a nine-fold increase in *MYLK2* expression in asthmatic cells (Table 3), which indicates the role of TGFβ1 in increasing ASM cell contraction. Unexpectedly, TGFβ1 decreased *CAMK2D* expression in both the non-asthmatic and asthmatic cells (Table 3). ASM cells can generate mediators and growth factors. TGFβ1 treatment induced a four-fold increase in *TGFβ2* expression and suppressed *ADAM33* expression by half to a level comparable to baseline asthmatic ASMCs (Table 3). Taken together, these results indicate that TGFβ1 can drive gene expression changes in non-asthmatic cells to be more contractile and proliferative similar to asthmatics ASMCs.

In response to SFN (Table 4), *CCND1* expression was reduced by 78% and 319% in non-asthmatic and asthmatic ASMCs, respectively. Similarly, SFN treatment

decreased *PCNA* expression by 68% in non-asthmatic ASMCs and by 98% in asthmatic ASMCs (Table 4). This result suggests that SFN can inhibit cell proliferation (seen in Figure 3B) by decreasing the gene expression of *CCND1* and *PCNA*. Moreover, a significant decrease in *COL3A* expression in SFN-treated asthmatic ASMCs was observed (Table 4). Non-asthmatic ASMCs also showed a decreasing trend in *COL3A* expression in response to SFN, although the change was not statistically significant ($p=0.08$) (Table 4). The results indicated that SFN might suppress collagen synthesis in ASMCs. In addition, SFN decreased genes associated with ASM cell contraction. SFN treatment decreased *CAMK2D* (90% and 77%) and *MYLK2* (84% and 78%) in both non-asthmatic and asthmatic ASMCs, respectively (Table 4). For ASM cell growth factors and mediators, SFN reduced *ADAM33* and *TGFβ2* expression by 56% and 89%, respectively, in asthmatic ASM cells only (Table 4). To summarize, SFN, by decreasing the expression of ASM phenotypic genes to levels that are comparable to that of non-asthmatics, may reverse the aberrant cell phenotypes seen in asthmatics ASMCs. Consistent with the anti-proliferative effects of SFN, we observed a larger change in the reduction of ASM phenotypic gene expression levels in asthmatic ASMCs than in non-asthmatic ASM cells. Furthermore, we demonstrated that the modulation of ROS levels might either drive non-asthmatic ASMCs toward aberrant cell phenotypes seen in asthmatics or reverse the abnormal cell phenotypes of asthmatic ASMCs.

Table 3: TGFβ1 induced changes in ASM phenotypic genes in human ASMCs.

Gene Symbol	Non-asthmatic		Non-asthmatic + TGFB1		Asthmatic		Asthmatic + TGFB1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Cell Proliferation								
CCND1	1.10	0.13	0.74	0.12	2.00(n=2)	0.03	0.81	0.04
PCNA	1.00	0.03	0.86	0.07	0.89	0.09	0.66	0.01
Collagen Synthesis								
COL3A	1.00	0.02	2.00***	0.23	1.00(n=3)	0.14	1.70	0.24
Cell Contraction								
CAMK2D	1.00	0.07	0.59*	0.13	0.87	0.08	0.11^^	0.01
MYLK2	1.00	0.10	3.90**	0.84	1.20	0.18	10.00^^^	0.56
Growth Factors and Mediators								
ADAM33	1.00(n=5)	0.14	0.43***	0.05	0.61#	0.08	0.22	0.01
TGFβ2	1.00	0.06	4.30***	0.49	1.90	0.85	6.2^^	1.90

Note:
mRNA levels were measured by qPCR after TGFB1 exposure at 1ng/mL for 48 hours in non-asthmatic and asthmatic ASM cells .
Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the untreated non-asthmatic ASM cells.
Results expressed as mean values ± SEM, with 7-9 non-asthmatic and 4 asthmatic donors (otherwise noted).

Unpaired ttest used to examine the statistical difference between Non-asthmatic and asthmatics untreated samples.
# p<0.05, ## p<0.01 or ### p<0.001 comparing Non-asthmatic vs Asthmatic

One-way ANOVA with Bonferroni's multiple comparisons tests used to compare the statistical significance between treatment groups.
*p<0.05, **p<0.01 or ***p<0.001 comparing Non-asthmatic vs Non-asthmatic+TGFB1
^ p<0.05, ^^p<0.01 or ^^^p<0.001 comparing Asthmatic vs Asthmatic+TGFB1

Table 4: Sulforaphane induced changes in ASM phenotypic genes in human ASMCs.

Gene Symbol	Non-asthmatic		Non-asthmatic + SFN		Asthmatic		Asthmatic + SFN	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Cell Proliferation								
CCND1	0.93	0.20	0.15	0.06	3.95$^{\#\#}$	0.82	0.76$^{\wedge\wedge\wedge}$	0.22
PCNA	1.00	0.06	0.32***	0.07	1.20 (n=3)	0.08	0.22$^{\wedge\wedge\wedge}$	0.03
Collagen Synthesis								
COL3A	0.75	0.13	0.33	0.09	0.92	0.13	0.24$^{\wedge\wedge\wedge}$	0.05
Cell Contraction								
CAMK2D	0.96	0.08	0.06*	0.01	1.20	0.23	0.43$^{\wedge}$	0.22
MYLK2	0.89	0.23	0.05*	0.01	0.89(n=4)	0.20	0.11$^{\wedge}$	0.04
Growth Factors and Mediators								
ADAM33	0.81	0.14	0.48	0.08	0.85	0.13	0.29$^{\wedge\wedge}$	0.08
TGFB2	0.83	0.12	0.48	0.22	1.30$^{\#}$(n=4)	0.15	0.41$^{\wedge}$	0.17

Note:
mRNA levels were measured by qPCR after Sulforaphane (SFN) exposure at 30uM for 48 hours in non-asthmatic and asthmatic ASM cells.
Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the untreated non-asthmatic ASM cells.
Results expressed as mean values ± SEM, with 4-6 non-asthmatic and 5-8 asthmatic donors per group (otherwise noted).

Unpaired ttest used to examine the statistical difference between Non-asthmatic and asthmatics untreated samples.
$^{\#}$ p<0.05, $^{\#\#}$p<0.01 or $^{\#\#\#}$p<0.001 comparing Non-asthmatic vs Asthmatic

One-way ANOVA with Bonferroni's multiple comparisons tests used to compare the statistical significance between treatment groups.
*p<0.05, **p<0.01 or ***p<0.001 comparing Non-asthmatic vs Non-asthmatic+SFN
^ p<0.05, ^^p<0.01 or ^^^p<0.001 comparing Asthmatic vs Asthmatic+SFN

Alterations in citrate metabolism via NAD^+-dependent redox cycling associated with TET activity

Next, we sought to investigate the effects of oxidative stress on TET activation by measuring the production of alpha-KG and the key factors involved in isocitrate metabolism (Figure 4). At baseline, asthmatic ASMCs showed increased mRNA levels of sirtuin NAD^+-dependent deacetylases (*SIRT1* and *SIRT3)* and isocitrate dehyogenase (*IDH2*) (Figure 4A and B) compared with non-asthmatic ASMCs. This result suggests the asthmatic ASMCs may have increased citrate metabolism compared with non-asthmatic ASMCs. First, we determined if increased ROS by TGFβ1 can alter alpha-KG production. We found that TGFβ1 had no effect on *SIRT1, SIRT3*, and *IDH1* in non-asthmatic or asthmatic ASMCs (Figure 4A and B). However, in non-asthmatic ASMCs, TGFβ1 did increase *IDH2* mRNA level by 80%, a level close to that of untreated asthmatic ASMCs (Figure 4B). TGFβ1 also caused an additional induction of *IDH2* (~300%) in asthmatic ASMCs (Figure 4B). In addition, the level of alpha-KG was increased by 70% in non-asthmatic ASMCs and no effect in asthmatic cells (Figure 4C). Consequently, the increased alpha-KG level in non-asthmatic ASMCs was associated with a 40% increase in TET activity (Figure 4D). Taken together, these findings indicated that TGFβ1 increased *IDH2* gene expression and subsequent alpha-KG production to up-regulate TET activity in ASMCs. Notably, TGFβ1 increased IDH2-mediated alpha-KG production and TET activity in non-asthmatic ASMCs to a level equivalent to that of untreated asthmatic ASMCs.

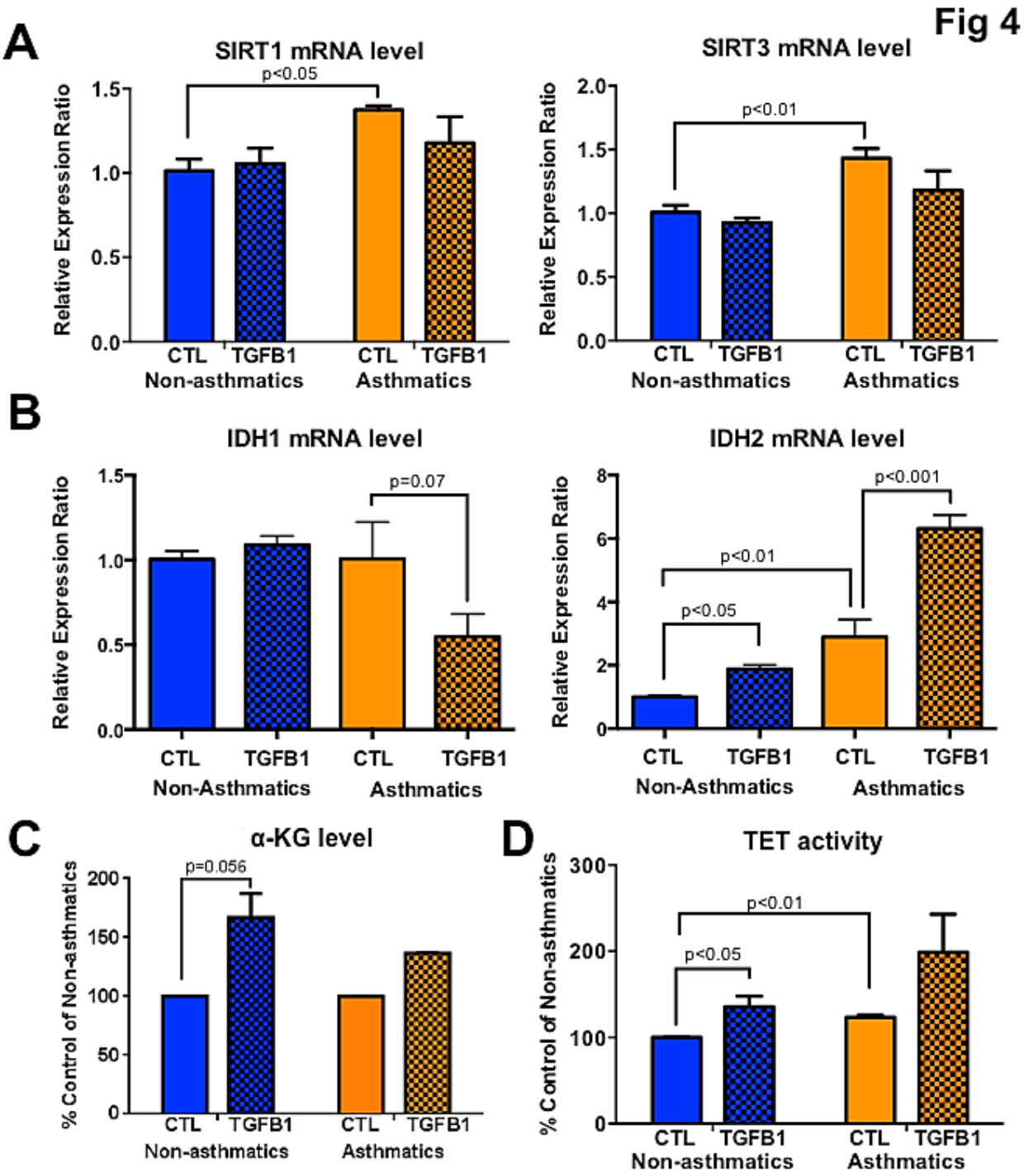

Figure 4. TGFβ1 increased IDH2 mRNA level and increased alpha-KG level and TET activity in non-asthmatic ASM cells. Cells harvested at 48 hours after TGFβ1 (1ng/mL) were subject to the measurement of **(A)** NAD-dependent deacetylase sirtuins (SIRT1/2) mRNA **(B)** Isocitrate dehydrogenase (IDH1/2) mRNA **(C)** alpha-ketoglutarate (alpha-KG) and **(D)** TET activity. **(A and B)** mRNA levels of SIRT1, SIRT2, IDH1 and IDH2 were measured by qPCR. Relative Expression Ratio (RER)

values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 6 non-asthmatic and 4 asthmatic donors. Two-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted. **(C)** alpha-KG level was measured by alpha-KG enzymatic assay kit. Relative change of alpha-KG production was normalized to the average of alpha-KG production of the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 3 non-asthmatic and 2 asthmatic donors per group. Two-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted. **(D)** TET activity was measured by ELISA. Relative change of TET activity in treated group was normalized to the TET activity of the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 4 non-asthmatic and 3 asthmatic donors per group. Multiple t-tests were used and statistically significant comparisons were noted.

Second, we investigated if the reduction of ROS levels by SFN can decrease TET activity *via* NAD+-dependent alpha-KG production. SFN treatment dramatically decreased (>60%) the expression of citrate metabolism genes, *SIRT1* and *SIRT3,* in asthmatic ASMCs to levels below those of the non-asthmatic and asthmatic controls (Figure 5A). In addition, *IDH1* and *IDH2* were down-regulated (>50%) in both non-asthmatic and asthmatic ASMCs treated with SFN (Figure 5B). In response to SFN, the *IDH2* mRNA levels in asthmatic ASMCs were reduced to levels comparable with those of non-asthmatic controls (Figure 5B). Furthermore, we showed that the reduction in *SIRT1, SIRT3, IDH1,* and *IDH2* mRNA levels was associated with decreased alpha-KG production and TET activity. SFN decreased alpha-KG levels in non-asthmatic and asthmatic ASMCs by 25% and 35%, respectively (Figure 5C). Consequently, we demonstrated that SFN can reduce TET activity in non-asthmatic and asthmatic ASMCs by 60% and 55%, respectively (Figure 5D). Therefore, our data suggested that SFN decreased citrate metabolism (*SIRT1, SIRT3, IDH1*, and *IDH2*) to abrogate alpha-KG levels. Decreased alpha-KG resulted in the reduction of TET activity. To summarize, although the increased ROS levels induced by TGFβ1 had no effect on NAD+-dependent deacetylases (*SIRT1 and SIRT3*), it did correlate well with increased *IDH2*-mediated alpha-KG production and TET activity in non-asthmatic ASMCs. By contrast, the decreased ROS levels by SFN comprehensively decreased citrate metabolism (*SIRT1, SIRT3, IDH1*, and *IDH2*) and accompanied diminished alpha-KG production and TET activity in both non-asthmatic and asthmatic ASMCs. The results suggest that intracellular alpha-KG levels are critical to modulating TET activity, and alpha-KG

production is ROS dependent. We summarized our findings in a schematic diagram shown in Figure 6.

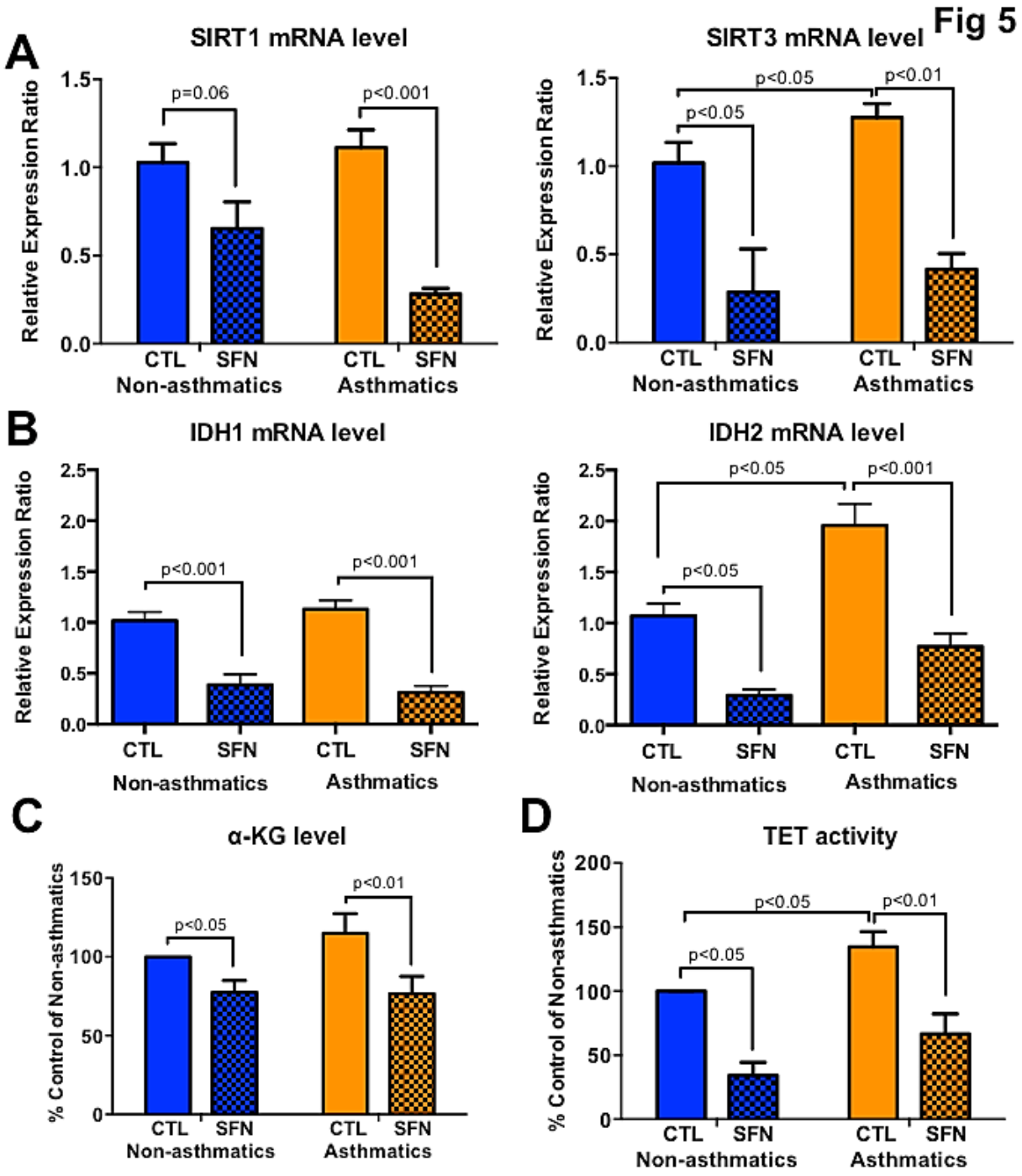

Figure 5. SFN decreased SIRT1, SIRT3, IDH1, IDH2 mRNA level, alpha-KG level and TET activity in asthmatic ASM cells. Cells harvested at 48 hours after exposure of Sulforaphane (SFN) (30uM) were subject to measurement of **(A)** NAD-

dependent deacetylase sirtuins (SIRT1/2) mRNA level, **(B)** Isocitrate dehydrogenase (IDH1/2) mRNA levels and **(D)** TET activity. (**C**) Assay for alpha-ketoglutarate (alpha-KG) level was performed at 24 hours after the SFN exposure. (A and B) mRNA levels of SIRT1, SIRT3, IDH1 and IDH2 were measured by qPCR. Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 4-6 non-asthmatic and 4-6 asthmatic donors per group. Two-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted. **(C)** alpha-KG level was measured by alpha-KG enzymatic assay kit and the percentage of alpha-KG levels were calculated compared to the average of the non-asthmatic control (CTL) group. Relative change of alpha-KG production was normalized to the average of alpha-KG production of the non-asthmatic control (CTL) group. Results expressed as mean values ± SEM, with 3 non-asthmatic and 3 asthmatic donors per group. Two-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted. **(D)** TET activity was measured by ELISA. Relative change of TET activity in treated group was normalized to the TET activity of the non-asthmatic control (CTL) group. Results are expressed as mean values ± SEM, with 3 non-asthmatic and 3 asthmatic donors per group. Two-way ANOVA with Bonferroni's multiple comparisons tests were used and statistically significant comparisons were noted.

Fig 6

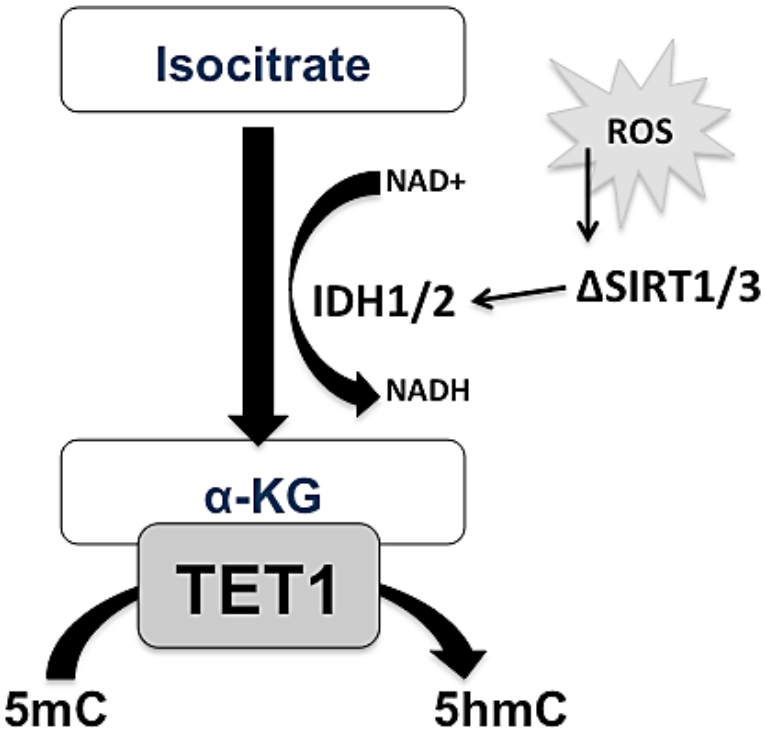

Figure 6. Proposed role of oxidative stress upon citrate metabolism and TET activity. Increased reactive oxygen species (ROS) in human ASM cells may affect NAD^+ cycling, which in turn activates the NAD-dependent deacetylase sirtuins (SIRT1/SIRT2) and Isocitrate Dehydrogenase (IDH1/IDH2) activity to increase the conversion of isocitrate to alpha-KG; a citrate metabolism intermediate. The resulting increased alpha-KG production, a cofactor for TET1, promotes TET1 enzymatic activity and the oxidation of 5mC to 5hmC. Our results indicated that modulation of ROS by TGFβ1 or Sulforaphane could modulate the level of ROS production, which in turn alters the citrate metabolism and eventually the TET activity.

Can oxidative stress alter epigenetic mechanisms other than TET1?

In addition to altered TET activity in ASMCs, changes in oxidant levels may trigger other DNA methylation machinery. In DNA methylation, DNMT1 maintains CpG methylation during replication, whereas both *MBD1* and *MECP2* are methyl-CpG binding domain (MBD) proteins that bind methylated DNA and act as transcriptional repressors. Both *TET1* and *TET2* are involved in DNA hydroxymethylation, an intermediate in the DNA demethylation pathway that requires DNA repair enzymes, such as *AID, MBD4*, and *TDG*. DNA deaminase *AID* removes an amino group from 5hmC to form 5-hydroxymethyluracil (5hmU), and DNA glycosylase enzymes *MBD4* and *TDG* mediate the removal of thymine, uracil, and 5-fluorouracil in G:T and G:U mismatched base pairs to generate the correct G:C base pairs. We investigated the gene expression of epigenetic modulators in ASMCs treated with TGFβ1 (Table 5). At baseline, asthmatic ASMCs showed significantly increased mRNA levels of *MBD1* and *TDG* compared with non-asthmatic ASMCs (Table 5). Asthmatic ASMCs also showed increased expression of *MBD4*, *TET1*, and *TET2* and decreased expression of *DNMT1*, although the changes were not statistically significant (Table 5). No significant changes in *DNMT1, MBD1*, and *MECP2* in non-asthmatic and asthmatic ASMCs treated with TGFβ1 were observed. In non-asthmatic ASMCs, TGFβ1 treatment markedly increased *MBD4* (50%), *TET1* (100%), and *TET2* (50%) expression to a level comparable to that of untreated asthmatic controls (Table 5). Also, a 90% increase in *AID* expression (although it was not statistically significant, p=0.059) was observed in non-asthmatic ASMCs treated with TGFβ1 (Table 5). Only *TDG* showed further increase by 190% in asthmatic ASMCs after exposure to TGFβ1 (Table 5). In summary, the results indicated that TGFβ1 modulates

the expression of enzymes involved in DNA demethylation machinery (*AID*, *TDG*, *MBD4*, *TET1*, and *TET2*) in non-asthmatic cells. How these methylation changes link to aberrant ASMC phenotypes requires further investigation.

Next, we sought to determine if the reduction of ROS levels modified the gene expression levels of DNA methylation/demethylation modulators (Table 6). No significant changes were observed in *DNMT1*, *MBD1, MECP2, TDG*, and *TET2* in non-asthmatic ASMCs treated with SFN (Table 6). By contrast, SFN significantly decreased *MBD4* and *TET1* expression, whereas it increased *AID* expression in both non-asthmatic and asthmatic ASMCs (Table 6). Only in asthmatic ASMCs did SFN decrease *MBD1* and *TET2* expression by 90% and 107%, respectively (Table 6). No significant changes in *TDG* expression were found in asthmatic ASMCs exposed to SFN (Table 6). To summarize, suppression of ROS levels by SFN can modulate the expression of genes involved in DNA demethylation (*MBD4*, *TET1*, and *AID*) in both non-asthmatic and asthmatic ASMCs. In addition, *MBD1* and *TET2* in asthmatic ASMCs were responsive to SFN treatment. Notably, SFN can reverse the aberrant epigenetic changes (at least DNA methylation machinery) in asthmatic ASMCs. How these methylation changes link to aberrant ASMC phenotypes also requires further investigation.

Table 5: TGFβ1 induced changes in mRNA levels of epigenetic modulators in human ASMCs.

Gene Symbol	Non-asthmatic		Non-asthmatic + TGFB1		Asthmatic		Asthmatic + TGFB1	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
DNA Methylation								
DNMT1	1.00	0.10	0.82	0.11	0.87(n=3)	0.04	0.56(n=3)	0.04
MBD1	0.85	0.05	1.24	0.20	1.54###	0.09	1.44	0.31
MECP2	1.00	0.07	1.20	0.05	1.20	0.07	0.94	0.08
DNA Hydroxymethylation								
TET1	0.87	0.10	1.9*	0.24	0.97	0.04	1.10	0.29
TET2	1.10	0.16	1.6*	0.11	1.50	0.17	1.90	0.12
DNA Repair								
AID	1.00(n=4)	0.16	1.90(p=0.06)	0.19	0.98(n=2)	0.37	1.60	0.27
MBD4	1.00	0.12	1.50*	0.08	1.20	0.06	1.40	0.08
TDG	1.00	0.05	3.3***	0.26	1.30#	0.09	3.20^^^	0.17

Note:

mRNA levels were measured by qPCR after TGFB1 exposure at 1ng/mL for 48 hours in non-asthmatic and asthmatic ASM cells .

Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the untreated non-asthmatic ASM cells.

Results expressed as mean values ± SEM, with 5-6 non-asthmatic and 4 asthmatic donors per group (otherwise noted).

Unpaired ttest used to examine the statistical difference between Non-asthmatic and asthmatics untreated samples.

# p<0.05, ##p<0.01 or ###p<0.001 comparing Non-asthmatic vs Asthmatic

One-way ANOVA with Bonferroni's multiple comparisons tests were used.

*p<0.05, **p<0.01 or ***p<0.001 comparing Non-asthmatic vs Non-asthmatic+TGFB1

^ p<0.05, ^^p<0.01 or ^^^p<0.001 comparing Asthmatic vs Asthmatic+TGFB1

Table 6: Sulforaphane induced changes in mRNA levels of epigenetic modulators in human ASMCs.

Gene Symbol	Non-asthmatic		Non-asthmatic + SFN		Asthmatic		Asthmatic + SFN	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
DNA Methylation								
DNMT1	0.97	0.15	0.91	0.26	0.79	0.06	0.26	0.07
MBD1	0.97	0.07	0.64	0.14	1.30$^{\#}$	0.11	0.41$^{\wedge\wedge\wedge}$	0.11
MECP2	0.98	0.06	3.50	1.70	1.10	0.11	1.80	0.25
DNA Hydroxymethylation								
TET1	1.03	0.09	0.32*	0.09	1.47$^{\#}$	0.19	0.44$^{\wedge\wedge\wedge}$	0.18
TET2	1.10	0.08	0.57	0.16	1.30	0.14	0.23$^{\wedge\wedge\wedge}$	0.08
DNA Repair								
AID	1.02	0.14	3.31***	0.16	1.20$^{\#\#}$	0.11	2.09	0.32
MBD4	1.00	0.09	0.40**	0.15	1.10	0.11	0.45$^{\wedge\wedge}$	0.06
TDG	0.98	0.07	0.97	0.16	1.10	0.04	0.72	0.12

Note:

mRNA levels were measured by qPCR after Sulforaphane (SFN) exposure at 30uM for 48 hours in non-asthmatic and asthmatic ASMCs.

Relative Expression Ratio (RER) values were calculated by 2^{-ddCt} method, where the RER was set as 1.00 in the untreated non-asthmatic ASMCs.

Results expressed as mean values ± SEM, with 4-6 non-asthmatic and asthmatic donors per group.

Unpaired ttest used to examine the statistical difference between Non-asthmatic and asthmatics untreated samples.

$^{\#}$p<0.05, $^{\#\#}$p<0.01 or $^{\#\#\#}$p<0.001 comparing Non-asthmatic vs Asthmatic

One-way ANOVA with Bonferroni's multiple comparisons tests were used.

*p<0.05, **p<0.01 or ***p<0.001 comparing Non-asthmatic vs Non-asthmatic+SFN

^ p<0.05, ^^p<0.01 or ^^^p<0.001 comparing Asthmatic vs Asthmatic+SFN

Sulforaphane pre-treatment protects mice from HDM-induced AHR

Finally, we explored a potential therapeutic strategy of targeting TET activity by administering antioxidants to relieve airway hyperresponsiveness (AHR) in an allergic AHR model. We investigated the *in vivo* effects of SFN upon HDM-induced AHR. HDM-exposed mice that were administered SFN showed decreased induction of airway resistance toward 10–30 mg/mL Mch by 40%–50% compared with the HDM-exposed mice, a result indicating that SFN exposures protected the mice from HDM-induced AHR (Figure 7A). In addition, SFN exposures can completely reverse the induction of alpha-KG production (Figure 7B) and TET activity (Figure 7C) by HDM in the mouse lung. Therefore, *in vivo*, we confirmed our findings from the human ASMCs that the suppression of ROS levels can decrease AHR phenotypes by modulation of alpha-KG production and TET activity.

Fig 7

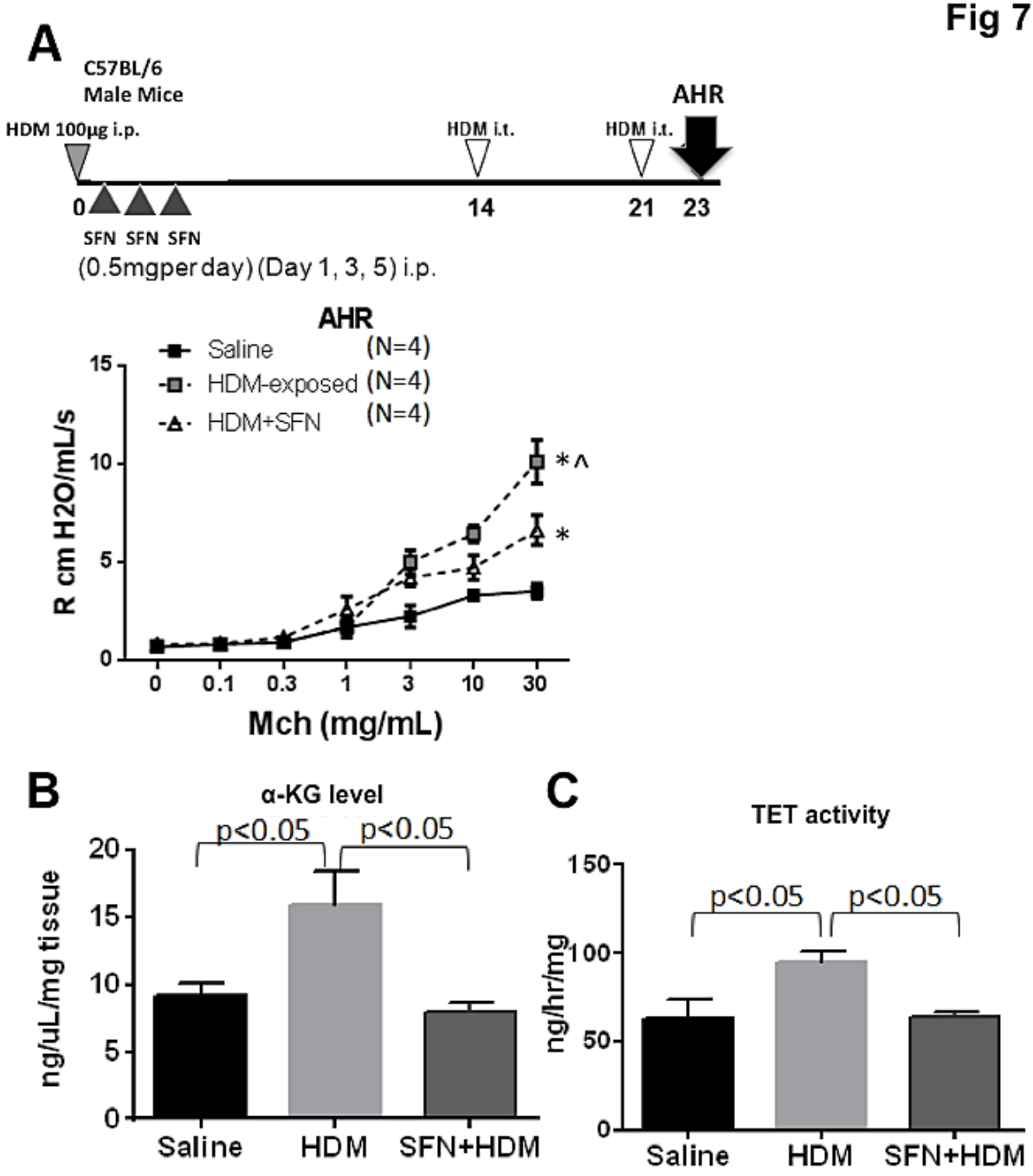

Figure 7. Sulforaphane treatment protects mice from HDM-induced AHR. **(A)** *Upper panel:* Schematic picture depicting the HDM sensitization and challenges used in this study. *Lower panel:* Methacholine (Mch) was administered to the animals at increasing doses (0.1, 0.3, 1, 3, 10, 30 mg/mL) by a 10 second aerosol inhalation. AHR was assessed as the change in pulmonary resistance (R, cm H20/mL/s) compared to baseline after the challenge to Mch. Results are expressed as mean values ± SEM with 4 mice per group; black box: Saline-exposed, grey box: HDM-exposed, triangle: HDM+SFN-exposed. Two-way ANOVA with Bonferroni's multiple comparisons test

was used to determine the p value, which were *$p<0.05$ vs Saline-exposed group; and ^ $p<0.05$ against HDM+SFN-exposed group. **(B)** alpha-ketoglutarate (alpha-KG) levels in lung tissue were measured by the alpha-KG enzymatic assay. Results expressed as mean values ± SEM, with 4 animals per group. One-way ANOVA with Bonferroni's multiple comparisons tests was used and statistically significant comparisons were noted. **(C)** TET activity was measured by ELISA. Results are expressed as mean values ± SEM, with 4 animals per group. One-way ANOVA with Bonferroni's multiple comparisons tests were used and statistically significant comparisons were noted.

DISCUSSION

Understanding TET1 regulation provides strategies to reverse the aberrant phenotypes of asthmatic ASMCs

In this study, we demonstrated that modulation of oxidant balance can alter TET activity and its-mediated ASMC phenotypes. As reported in other studies, increased oxidative stress has been shown to be associated with the increased airway inflammation and reactivity of asthmatic patients (9–12). Increased oxidative stress in asthma patients affects many cells in the airways. For example, ROS production has been reported from epithelial, type II alveolar, endothelial, and smooth muscle cells in the airways generated from mitochondrial stress, NADPH enzymes, and inflammatory cells (121). In ASM cells, the oxidative stress burden is mostly attributed to increased NOX4 expression in asthmatics, which is correlated with the increased ASMC contraction mitigated in the presence of NOX4 inhibitors (123, 127). In addition, human asthmatic ASMCs exhibited decreased nuclear factor (erythroid-derived 2)-like 2 (NRF2) binding to the antioxidant response element (ARE), as well as decreased heme oxygenase (HO)-1 mRNA and protein levels, the primary anti-oxidant defense pathway activated from NRF2 activation, compared with non-asthmatic controls (128). These results indicate the repressive role of anti-oxidant defense mechanisms in asthmatic ASMCs. Therefore, modulating the oxidant balance in ASMCs can play a part in the regulation of ASMC phenotypes observed in asthma pathogenesis. Our current findings support this idea by demonstrating the effect of TGFβ1 and SFN on ASMCs. Furthermore, we showed that the regulation of ASMC phenotypes by ROS is facilitated at least partly thorough epigenetic modifications. We used TGFβ1 and SFN as a pro-oxidant and anti-oxidant, respectively, to modulate the levels of oxidative stress in ASMCs. We showed that TGFβ1 and SFN

had opposing effects on modifying ROS levels in the human ASMCs model. TGFβ1 is considered an immunomodulator in asthma pathogenesis and can lead to adverse effects in animal models of asthma as both a potent suppressor of inflammation, as well as an inducer of AHR with increased fibrosis, inflammation, and airway remodeling (129). In addition, TGFβ1 induced gene expression of activating transcription factor (*ATF3*), an *NRF2* repressor, whereas it decreased the gene expression of *HO-1* in human ASMCs (128). These previous studies validate our current findings that TGFβ1 increased ROS levels, possibly through the repression of NRF2 activation and subsequent decrease in phase II enzymes. Moreover, TGFβ1 is continually up-regulated in asthmatic patients and has physiologic relevance to airway remodeling in asthma pathogenesis (125). On the other hand, SFN is a phytochemical abundant in broccoli extracts and is a potent inducer of endogenous phase II detoxification enzymes that scavenge reactive oxygen species to mitigate oxidative stress and maintain oxidative homeostasis (130).

We showed that TGFβ1 induced the proliferative phenotypes in ASMCs, whereas SFN suppressed the aberrant ASMC phenotypes seen in asthmatics. We and others have shown that asthmatic ASMCs proliferate more, contract more, and synthesize collagen more than non-asthmatic ASM cells (102). In addition, we reported that asthmatic ASMCs have increased ROS levels compared to non-asthmatic cells (Figure 2). *Thus, we examined whether increased ROS levesl by TGFβ1 in non-asthmatic cells can induce phenotypic changes in non-asthmatic ASM cells to resemble asthmatic ASMCs and vice versa with SFN treatment on asthmatic ASMCs to reverse the aberrant ASMC phenotype.* We found that TGFβ1 increased cell proliferation in non-asthmatic cells, while SFN treatment decreased cell proliferation in both non-asthmatic and asthmatic cells,

measured by the BrdU incorporation assay (Figure 3A). Furthermore, we demonstrated that molecular changes are linked with aberrant ASM phenotypes by measuring the expression of genes related to cell proliferation, cell contraction, and synthesis of collagen, growth factors, and mediators in ASMCs. Exposure to TGFβ1 in non-asthmatic ASMCs induced the expression of *COL3A*, *MYLK2*, and *TGFβ2*, a result suggesting that TGFβ1 may drive non-asthmatic ASMCs to produce collagen, contract more, and grow faster than asthmatic ASMCs.

By contrast, reduction of ROS level by SFN reduced cell proliferation (Figure 3B) in both non-asthmatic and asthmatic ASMCs. Moreover, SFN decreased the expression of genes involved in cell proliferation (*CCND1, PCNA,* and *TGFβ2),* collagen synthesis *(COL3A)*, and cell contraction *(CAMK2D* and *MYLK2)* in asthmatic ASMCs. Our results are consistent with previous *in vitro* investigations in human ASMCs that reported SFN induced *NRF2* protein expression and ARE activation to reduce ASMC proliferation (128). Our findings indicated that SFN can possibly reverse the asthmatic ASMC phenotypes and induce asthmatic cells to appear phenotypically non-asthmatic. Most impressive, we found that asthmatic ASMCs treated with SFN had a significant reduction in cell proliferation and expression of ASM phenotypic genes to levels below the baseline of untreated non-asthmatic ASMCs. This result might be due to the fact that NRF2 activity or other NRF2-signaling pathways in asthmatic ASMCs are susceptible in response to antioxidant defense, such as SFN exposure. However, the significant suppressive effects of SFN upon cell proliferation could also be related to the dose of SFN exposure.

Moreover, the effects of SFN treatment on modulating ASM-related phenotypes were corroborated with our *in vivo* model demonstrating that SFN exposure can reduce allergic–AHR and is associated with a decrease in TET activity in mouse lungs (Figure 7). In addition, we showed that SFN exposure can reverse the induction of TET activity in asthmatic ASMCs (Figure 5). Given the fact that inhibition of *TET1* can reverse the aberrant ASM cell phenotypes and HDM-induced AHR (Chapter 2), our present findings support the use of SFN to reduce airway oxidative stress in the therapeutic treatment of asthma. Indeed, clinical studies have shown that the administration of oral SFN in broccoli sprout extract homogenate induced phase II enzymes glutathione-s-transferase M1 (*GSTM1*), glutathione-s-transferase P1 (*GSTP1*), NADPH quinone oxidoreductase (*NQO1*), and hemoxygenase-1 (*HO-1*) in the upper airway of human subjects compared with the controls, who ingested alfalfa sprouts, measured by qPCR analysis in nasal lavage cells (130). Additionally, oral broccoli sprout extract consumption in allergic asthma subjects for four days attenuated the induction of total cell counts in the nasal lavage after diesel exhaust particle challenge (131). Taken together, these results support the therapeutic and nutritional value of SFN consumption to ameliorate oxidative stress in the lungs. Although the therapeutic potential of SFN treatment in airway inflammation has already been investigated, our study is the first to associate SFN with decreased alpha-KG production and decreased TET enzymatic activity, especially in ASMCs that can suppress asthmatic ASMC phenotypes.

Novel role of ROS in citrate metabolism and subsequent TET activity of ASMCs

We identified a novel role of ROS in regulating epigenetic modifications *via* alterations in citrate metabolism. Our asthmatic ASMCs had increased TET activity and

ROS levels, a result suggesting that TET activity in ASMCs is likely regulated through oxidative stress pathway. We hypothesized that the oxidative stress level regulates citrate metabolism to modify alpha-KG production and subsequently activate TET enzymes. All TET proteins (*TET1-3*) contain catalytic domains at their carbonyl-terminus, which consist of both a cysteine-rich region and dioxygenase, which converts 5mC into 5hmC, with the use of Fe^{2+} and alpha-KG. Under oxidative stress conditions, the level of NAD^{+} was increased in the mitochondria, which can activate the SIRT family of protein deacetylases, which in turn deacetylate IDH2 to promote IDH2 activity (20, 132). We demonstrated that TGFβ1 increased alpha-KG production and TET activity in non-asthmatic ASMCs. Only *IDH2* gene expression was increased, and no significant effects were found on *SIRT1, SIRT3*, and *IDH1* gene expression (Figure 4). These findings suggest that induction of alpha-KG production and TET activity by TGFβ1 may be dependent on IDH2 activity. Further experiments will be performed to examine the link between TGFβ1-induced ROS production and IDH2-mediated alpha-KG production. Nonetheless, our findings indicate the possible role of ROS production in TET up-regulation in asthmatics (summarized in Figure 6).

In addition, we demonstrated that SFN significantly decreased the gene expression of *SIRT1, SIRT3, IDH1*, and *IDH2*, which subsequently reduced intracellular alpha-KG level and TET activity in both non-asthmatic and asthmatic ASMCs (Figure 5). Furthermore, these findings support our hypothesis that SFN may apply as an epigenetic modifier to suppress TET activity and reverse the *TET1*-mediated expression of ASM phenotypic genes reported earlier. In this study, we focused on the effect of ROS on alpha-KG production given the fact that we found up-regulation of *SIRT1, SIRT3, IDH1*,

and *IDH2* in asthmatic ASMCs. Nevertheless, modulation of Fe^{2+} level by ascorbic acid was shown to enhance TET activity (133). Low plasma levels of ascorbic acid have also been reported to be associated with increased asthma risk. However, the link between ascorbic acid and TET-mediated asthma pathogenesis has not yet been identified. As a first step, we demonstrated that ascorbic acid did not alter TET activity in ASMCs, a result suggesting that the mechanisms underlying TET regulation may be tissue or cell specific. Also, since TET proteins are 2-Oxoglutarate-dependent-oxygenases that require 2-Oxoglutarate (alpha-KG) to convert 5mC to 5hmC (52, 134) , we further provided the evidence that alpha-KG plays a critical role in regulating TET enzymes in ASMCs.

ROS may also mediate epigenetic changes via TET-independent mechanisms, such as DNA or histone methylation that can be modulated by GSH depletion (135, 136). Therefore, we investigated the roles of other epigenetic modulators involved in DNA methylation machinery in modifying epigenetic changes in ASMCs in response to oxidative stress. TGFβ1 induced the gene expression of *TET1, TET2*, *AID*, *MBD4*, and *TDG* (DNA hydroxymethylation and DNA repair-mediated demethylation) in non-asthmatic ASMCs that might contribute to the aberrant alterations in ASM phenotypic gene expression and ASM phenotypes. No significant changes in the expression of modulators responsible for DNA methylation were observed (*DNMT1*, *MBD1*, and *MECP2*). These results suggest that increased ROS by TGFβ1 may promote DNA demethylation/hydroxymethylation rather than suppress DNA methylation machinery. They are also consistent with our previous findings that exposures to allergen induced a profile of demethylated candidates in mouse lung (45).

Asthmatic ASMCs, in response to SFN, showed a decreased expression of *TET1, TET2*, and *MBD4* (DNA hydroxymethylation and DNA repair-mediated demethylation). These results provide evidence that a reduction of ROS levels by SFN suppressed DNA hydroxymethylation and demethylation. By contrast, SFN exposure induced *MECP2* expression in ASMCs, a result suggesting that SFN may facilitate DNA methylation. Although we have not identified any gene-specific changes associated with changes in the expression of these epigenetic modulators, these preliminary findings provide a general understanding of the role of ROS in epigenetic regulation. The use of SFN has been shown to modulate a wide range of epigenetic modifications, including the hypomethylation of the *NRF2* promoter, decreased DNMT1 and DNMT3A protein levels, and decreased HDAC1,4,5,6 protein levels, which improve prostate cancer treatment (126). Altogether, our investigation into the alterations of epigenetic modulators in response to TGFβ1 and SFN provides insight into the mechanisms underlying epigenetic modulation of ASM phenotypes and subsequent AHR phenotypes. Moreover, it provides possible therapeutic tools to target the ROS-dependent citrate metabolism enzymes and *TET1*-mediated DNA hydroxymethylation.

Citrate metabolism and DNA methylation in ASMCs

In this study, we showed the effect of ROS on alpha-KG production and its subsequent TET activation in ASMCs. We demonstrated that oxidative stress modulated the generation of alpha-KG from isocitrate by SIRTs and IDH. During glycolysis, glucose is converted to pyruvate, which is in turn converted to acetyl-CoA by the pyruvate dehydrogenase complex in an oxidative process in which NADH and CO^2 are formed. Acetyl-CoA enters the Krebs cycle when it converts to citrate and subsequently

to isocitrate and alpha-KG. Alternatively, glycolysis also generates serine, an essential amino acid and precursor for purine and pyrimidine biosynthesis (137). Serine not only provides the methyl groups critical to the synthesis of DNA nucleotides but also contributes methyl groups to the increased formation of S-adenosylmethionine (SAM) substrates, which are the required methyl donors for DNMT enzymes to maintain DNA methylation (137). Increased serine production generates alpha-KG as a by-product of the phosphoserine aminotransferase reaction (137). Therefore, the generation of alpha-KG metabolite pools occurs through diverse metabolic regulation pathways and can alter epigenetic profiles through increased TET or DNMT activity. For example, in cancer cells, the increased glucose uptake is metabolically reprogrammed, often called the Warburg effect, to divert from the citric acid cycle, and it instead increases in serine metabolism to promote anabolism and proliferation, which increases the SAM and alpha-KG metabolite levels to alter DNA methylation *via* DNMTs and TETs. These results indicate that intracellular pools of metabolites, such as alpha-KG, can have important implications on the regulation of many epigenetic modulators implicated in disease phenotypes.

In the present study, we have not shown that alpha-KG production is dependent on isocitrate metabolism because the generation of alpha-KG production in cells can be done in many ways. In the future, we aim to confirm that the changes in alpha-KG production from TGFβ1 and SFN exposure are mediated through citrate metabolism pathways by suppression of IDH activity. Moreover, which oxidative stress defense pathways modulate the alpha-KG metabolite levels in ASMCs will also be examined. Our current study reported the global associations between alpha-KG production and

increased *TET1* activity. Further investigations on the gene-specific changes in DNA methylation and hydroxymethylation through ROS-dependent TET activity are warranted. Finally, to convincingly prove that *TET1* is modified through oxidative stress to change ASMC phenotypes, we plan to determine if the repression and induction of *TET1* activity in ASMCs can modulate the cell proliferation phenotypes. In conclusion, our results suggest, for the first time, the association between oxidative stress level and alpha-KG production, which in turn alters TET activity and modifies ASM phenotypic gene expression and ASMC phenotypes in relation to asthma pathogenesis. Notably, the anti-oxidant effects of SFN significantly reduced oxidative stress-mediated alpha-KG production, diminished the proliferative phenotypes of ASMCs, and reduced AHR phenotypes *in vivo*. Our findings not only improve our understanding of epigenetic regulation in asthma pathogenesis but also provide insight into the design for epigenetic modifiers to reverse aberrant AHR phenotypes.

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CHAPTER 4

CONCLUSIONS, PERSPECTIVES AND FUTURE STUDIES

4.1 Tet1-mediated DNA hydroxymethylation regulates allergic-induced airway hyperresponsiveness

The rise in the prevalence of asthma has increased the need for innovative research to understand the complex mechanisms underlying asthma pathogenesis. More comprehensive understanding of asthma etiology will improve prospective novel and effective therapeutic treatments. Given that asthma pathogenesis involves a gene-by-environment interaction, epigenetic mechanisms likely contribute to asthmatic disease. Therefore, unraveling the underlying epigenetic code contributing to asthma pathogenesis can highlight distinct epigenetic mechanisms responsible for aberrant biological pathways in asthma. Since epigenetic changes in the genome are reversible increased epigenetic research offers a tremendous therapeutic opportunity to target these unique mechanisms and possibly reverse or modify gene expression regulation and the pathogenesis of asthma.

Chronic HDM challenges in C57B6 mice increased airway hyperresponsiveness (AHR) that corresponded with increased Tet activity and 5hmC product in mouse lungs and airway smooth muscle cells (ASMCs). Thereafter, we hypothesized that Tet1-mediated hydroxymethylation contributes to allergen-induced AHR through epigenetic regulation of lung cells function. Using a heterozygous Tet1 deficient (*Tet1*$^{+/-}$) mouse model, we demonstrated that the loss of Tet1 protected mice from allergic AHR. Furthermore, Tet1 deficiency significantly reduced the HDM-induced global 5hmC content in both the lung and ASMCs. Additionally, the loss of Tet1 activity corresponded with the reversal of the HDM-induced gene expression of AHR-related genes in both the lung and ASMCs. Overall, these findings depict a novel role of Tet1

and its-mediated DNA hydroxymethylation upon global epigenetic regulation in the lung and tissue specific changes in ASMCs in response to environmental exposures to house allergen. To enhance our understanding of the role of ASM in asthma pathogenesis, we have to further investigate the role of aberrant ASM epigenetic regulation in regards to lung function by depleting *Tet1* specifically in mouse ASMCs, with a tissue-specific *Tet1* knockout mouse model. Also, it would be interesting to evaluate the Tet1-mediated epigenetic changes in the development of the inflammatory immune response with HDM-exposure, especially with repeated exposures to HDM. Hence, we envisioned using the Tet1 deficient mouse model to improve our understanding of asthma pathogenesis through identifying the role of environmental perturbations upon lung function phenotypes and epigenetic changes in the lung as well as in the diverse cell types involved in asthma pathogenesis; ASMCs, epithelial cells, and immune cells.

4.2 Targeting Tet1 activity could be applied for preventive or therapeutic approaches for treating asthma

In addition to the demonstration on the role of Tet1-mediated DNA hydroxymethylation in mouse AHR, we further confirmed the hypotheses generated in mouse systems in primary human asthmatic ASM cells. Certainly, asthma pathogenesis is complex with heterogeneous changes induced in the whole lung. While the changes that others and we have identified to date in the immune cell types are important and likely reflect changes in the airway inflammatory status of patients, few studies have examined epigenetic changes directly in airway resident cells like ASMCs. ASM remodeling (increased ASM mass and collagen synthesis by ASM) together with enhanced ASM contractility has been suggested to play an important role in AHR either

dependently or independently of airway inflammation. Therefore, we narrowed our focus on the epigenetic regulation of human ASMCs from non-asthmatic and asthmatic lungs. Asthmatic ASMCs had increased TET activity and 5hmC content as compared to non-asthmatic ASMCs. In addition, si*TET1* knockdown abrogated TET1 expression and TET activity; and attenuated the increased cell proliferation and expression of ASM phenotypic genes (*PCNA, SMA, CAMK2D, MLCK* and *TGFβ2*) in asthmatic ASMCs. Our conclusions our summarized below in Figure 1. These findings support the notion that TET1 regulates the asthmatic phenotypes of ASMCs *via* modulation of the expression of ASM phenotypic genes. In addition to gene expression changes in cell contraction genotypes, it would be worthwhile to investigate cell contraction phenotypes through optical magnetic twisting cytometry methods which measure ASM cell stiffness in response to a bronchiconstrictor and bronchidilator.

We further identified a novel role of oxidative stress in regulating TET activity in ASMCs. We demonstrated that airway oxidative stress levels could alter citrate metabolism and the generation of alpha-ketoglutarate (alpha-KG) metabolites, a co-factor critical for TET enzymatic activity. Applying immunomodulator TGFβ1 as a pro-oxidant, and Sulforaphane (SFN) as an anti-oxidant in both non-asthmatic and asthmatic ASMCs we found that TGFβ1 increased citrate metabolism and subsequent TET activation while SFN decreased citrate metabolism (*IDH1, IDH2, SIRT1* and *SIRT3*). Similarly, these changes in citrate metabolism corresponded to the reduction of alpha-KG production and TET activity. Consequently, TGFβ1 induced more proliferative phenotypes in non-asthmatic cells while SFN repressed the increased cell proliferation phenotype in asthmatic ASMCs. Taken together, these findings indicate that oxidative

stress levels in ASMCs play a significant role in the generation of alpha-KG metabolites, which regulate TET enzymatic activity and are linked to ASMCs phenotypes; summarized below in Figure 1. This mechanism of TET regulation warrants further investigation to understand which oxidative stress-related transcription factors and signaling pathways are perturbed in asthmatic ASMCs, which may contribute to increased level of reactive oxygen species, activity of citrate metabolism IDH enzymes, and alpha-KG production.

In conclusion, this thesis demonstrated a new mechanistic pathway of asthma pathogenesis in which environmental allergen exposure results in the epigenetic regulation of gene expression that modulates ASM function. We identified the role of TET1-mediated DNA hydroxymethylation upon AHR through elucidating the epigenetic regulation of ASMCs. Also, this work provides new insights into the understanding of asthma pathogenesis. Furthermore, we discovered that increased oxidative stress in asthmatic ASMCs increased citrate metabolism and the generation of alpha-KG metabolites resulting in the up-regulation of TET1 activity. Dysregulation of TET1 in ASMCs leads to persistent changes in the ASM cell phenotype in human asthmatics. These results are summarized below in Figure 1. Moreover, we demonstrated that antioxidant treatment may be effective in reversing the aberrant ASM cell phenotypes seen in asthmatics. We also showed administration to SFN could prevent the epigenetic modifications induced by allergen challenge and as such may prevent the long-term consequences of allergen sensitization including ASM remodeling and AHR. These results provide unique perspectives on the epigenetic signatures implicated in asthma pathogenesis and the possible use of anti-oxidants to modulate TET1-mediated DNA

hydroxymethylation to reverse asthmatic ASMC phenotypes. Furthermore, this thesis work provides a logical framework to investigate aberrant epigenetic mechanisms, in addition to TET1, and how these modifications link to physiopathological changes in the lungs. Overall, these results may lead to the development of new therapeutic approaches for the treatment of the asthma disease.

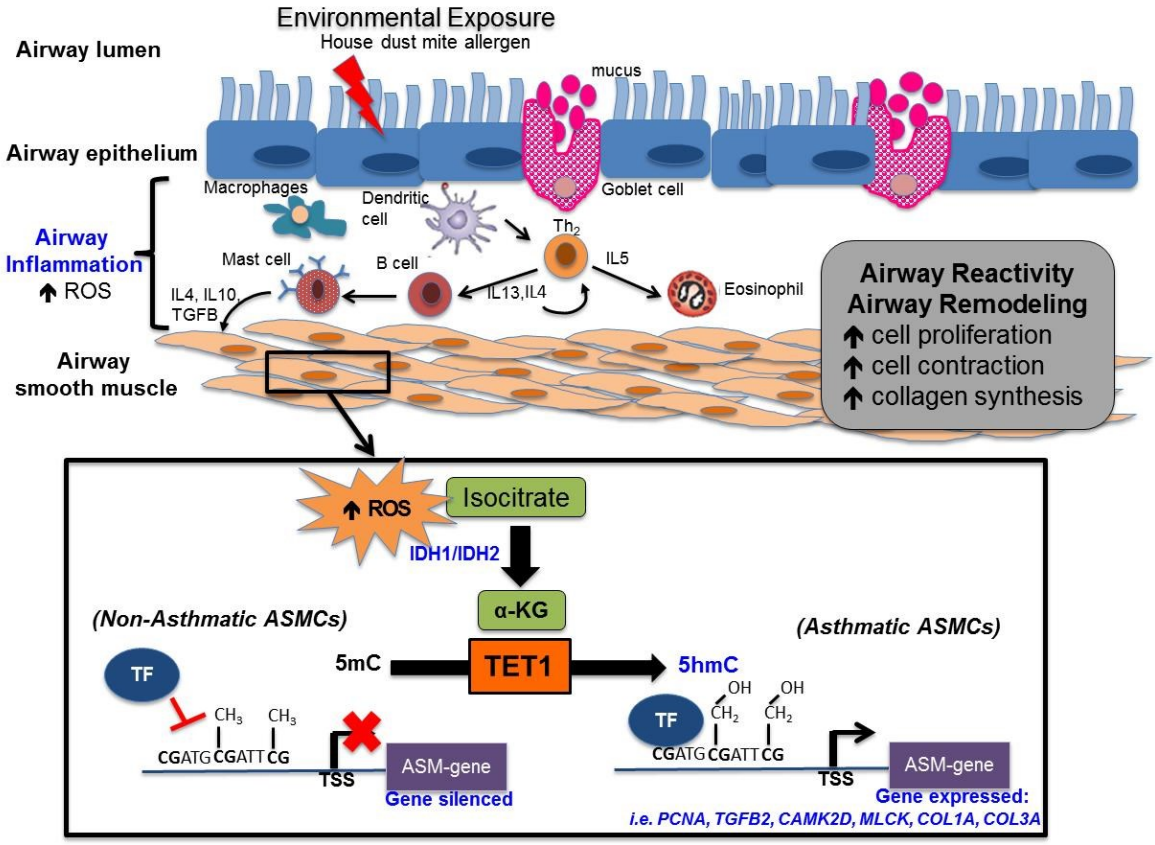

Figure 1. Proposed model of TET1-mediated DNA hydroxymethylation in the regulation of asthmatic ASM cells. Environmental exposures to allergens in the airway lumen can induce a pro-allergic Th2 mediated immune response with increased mast cell and eosinophils to increase both airway inflammation and oxidative stress. Asthmatic airways are characterized by imbalances in oxidative stress homeostasis; with increased reactive oxygen species (ROS) produced and decreased anti-oxidant enzymes to mitigate the damage. Increased airway oxidative stress can impact many cell types including the airway smooth muscle cells (ASMCs). We demonstrated that increased oxidative stress in ASMCs induced citrate metabolism, by increasing *IDH1* and *IDH2* expression, which increased the generation of a-KG, a required co-factor for TET1 enzymatic activity. Increased TET1 mediated the increase in 5hmC content. Increased TET1-mediated DNA hydroxymethylation in ASMCs corresponded with increased cell proliferation phenotypes and the increased gene expression of ASM cell proliferation genes (*PCNA,*

TGFβ2), cell contraction genes (*CAMK2D, MLCK*) and collagen synthesis genes (*COL1A, COL3A*). Therefore, our study provides evidence that the up-regulation of TET1 alters DNA methylation profiles to increase the gene expression of ASM phenotypic genes and alter the phenotypes of ASMCs to be more proliferative and contractile, features of asthmatic patients. These changes might persist and ultimately contribute to the epigenetic regulation of ASMCs.

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Tyna Dao

Education

Johns Hopkins Bloomberg School of Public Health 08/2011 – 09/2015
PhD in Department of Environmental Health Sciences (Physiology and Toxicology)
Certificate: Risk Sciences and Policy
Honors: Sommer Scholar

University of California Berkeley 08/2004 - 05/2008
B.S. in Molecular Toxicology

Publications

Balmer NV, **Dao T**, Leist M, Vojnits K, Waldmann T, Zhao L, Hogberg HT, van Vliet E. Application of "Omics" Technologies to In Vitro Toxicology. In Vitro Toxicology Systems. Methods in Pharmacology and Toxicology pp 399-432. 2014 Apr 09.

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Dao T, Limjunyawong N, Huang J, An S, Solway J, White S, Mitzner W, Tang W.Y. (2015) *Tet1-mediated hydroxymethylation and airway hyperresponsiveness*.

Limjunyawong N, Yeung B, **Dao T**, Huang J, An S, Mitzner W, Tang W.Y. (2015) *Overexpression of TGFB2 via DNA hydroxymethylation contributes to aberrant airway smooth muscle cell phenotypes in asthmatics.*

Dao T, Huang J, An S, Solway J, White S, Mitzner W, Tang W.Y. (2015) *Role of oxidative stress on TET1-mediated hydroxymethylation in human airway smooth muscle cells.*

Research Experience

Johns Hopkins School of Public Health 01/2013 – 09/2015
PhD Candidate -- Lab of Dr. Winnie Wan-Yee Tang
Baltimore, MD

- Examined the effects of allergen (house dust mite) exposure on lung function (airway hyperresponsiveness) through alterations in epigenetic mechanisms in mouse models.
- Translated animal findings into human relevance with targeted mechanistic studies in human airway smooth muscle cells *in vitro.*

Center for Alternatives to Animal Testing (CAAT) 05/2013 - 01/2015
Research Assistant -- Evidence Based Toxicology Collaborative (EBTC)
Baltimore, MD

- Developed and implemented an innovative systematic review, using Cochrane Review methods, to compare the chemical toxicity in mammalian and Zebrafish models for the improvement of toxicological decision-making

FDA – Center for Food Safety and Applied Nutrition 09/2009 - 06/2011
Toxicology Reviewer -- Office of Food Additive Safety
College Park, MD

- Reviewed and summarized, public and confidential, animal toxicity and genetic toxicity studies on direct and indirect food additives to update the internal agency PAFA (Priority-based Assessment of Food Additives) database.
- Initiated and implemented effective changes to the PAFA standard operating procedures.

FDA – Center for Drug Evaluation and Research 05/2008 - 07/2009
Computational Toxicologist -- Informatics and Computational Safety Analysis Staff
Silver Spring, MD

- Generated over 200 SAR (structure activity relationship) models based on post-market surveillance data from the FDA Adverse Event Reporting System and from published literature for ~1500 pharmaceuticals to predict 22 human adverse pulmonary toxicity endpoints.

University of California Berkeley School of Public Health 08/2006 - 05/2008
Research Assistant -- Gardening to Prevent Childhood Obesity, School of Public Health
Berkeley, CA

- Developed and instructed 40 minute lesson plans to emphasize goal setting, obesity and diabetes risk factors in a pilot study in middle school classes of 30 students.

Presentations

JHSPH 2015 EHS Departmental Retreat, Baltimore, MD 01/2015
TET1 Mediated Hydroxymethylation in Human Airway Smooth Muscle Cells (First Author)

JHSPH EHS Departmental Seminar, Baltimore, MD 09/2014
Epigenetic regulation in human airway smooth muscle cells: Role of TET1-mediated hydroxymethylation

American Thoracic Society 2014 Annual Meeting Poster, San Diego, CA 05/2014
Epigenetic Regulation of Transforming Growth Factor Beta2 (TGFB2) In Airway Smooth Muscle Cells (First Author)

2014 Delta Omega Scientific Poster Competition, Baltimore, MD 03/2014
Environmental Exposure To House Allergens Linked to 5hmC Perturbations In Lung (First Author) * Poster received 2nd place in the laboratory research section

JHSPH 2014 EHS Departmental Retreat, Baltimore, MD 01/2014
Environmental Exposure to House Allergens Linked to 5hmC Perturbations in Lung (First Author)

JHSPH 2013 EHS Departmental Retreat, Baltimore, MD 01/2013
PBDE Exposure in Cord Blood and DNA Methylation (First Author)

Poster Presented at the Society of Teratology Meeting, Baltimore, MD 06/2012
DNTox-21c Identification of Pathways of Developmental Neurotoxicity for High-throughput Testing by Metabolomics (First Author)

Posters Presented at the Society of Toxicology Convention, Baltimore, MD 03/2009

- *Adverse Effects of Pharmaceuticals: Construction of a Relational Database of Immunological and Pulmonary Adverse Effects Using FDA Archives, PharmaPendium™ and Public Sources* (First Author)
- *Prediction of Human Adverse Immunological and Pulmonary Effects using MC4PC, BioEpisteme, and Predictive Data Miner Software Programs* (Second Author)
- *Predicting Maximum Tolerated Dose Using Structure-based Similarity Searching and Dose vs. Exposure Time Data* (Second Author)

Computational Toxicology at the FDA Seminar, Berkeley, CA 02/2009

- Invited as a visiting lecturer for the undergraduate course in Molecular Toxicology at University of California Berkeley for three days.

Teaching Experience

Johns Hopkins Bloomberg School of Public Health 06/2012 - 11/2013

- Public Health Toxicology 4th Term 2013
- Environmental Health Summer 2013
- Environmental Health (Online) 3rd Term 2013
- Environmental Health Summer 2012